Unravelling the Complexity of the Molecular and Physiological Response to Environmental Change in Seagrasses

Thesis

Miriam Ruocco (M.Sc.)

Unravelling the complexity of the molecular and physiological response to environmental change in seagrasses

Doctor of Philosophy

School of Life, Health and Chemical Sciences

Open University UK

Stazione Zoologica Anton Dohrn
Department of Integrative Marine Ecology

Director of studies

Dr. Gabriele Procaccini

Stazione Zoologica Anton Dohrn
Department of Integrative Marine Ecology
Villa Comunale
80121 Napoli
Italy

External supervisor

Prof. Fabio Bulleri

Università di Pisa
Department of Biology
Unit of Ecology and Marine Biology
Via Derna 1
56126 Pisa
Italy

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Abstract

This thesis explores the complexity of seagrass stress response in the face of current environmental changes. This is a timely and relevant issue due to the role supplied by these foundation species in coastal ecosystems, and the dramatic consequences their loss would cause on marine biodiversity and human well-being.

Using as target species the iconic Mediterranean seagrass *Posidonia oceanica*, here I show: i) how molecular reprogramming, acting primarily at gene-expression level, coordinates physiological and morphological responses to different stressors, and ultimately determines species' acclimation strategies and tolerance capacity; ii) the differential stress response existing within and among different organs, and between different shoot types; iii) how the response to a single stressor can be modified depending on its temporal variability, and due to the interaction with another stressor.

In this study, new transcriptome data have been generated, from leaves and shoot-apical meristems, increasing considerably molecular resources available for future studies on seagrass evolutionary ecology and functional genomics. Moreover, this research sheds first light on the stress response of organs other than leaf, in seagrasses, and recognises the shoot meristem as a key determinant of whole plant survival.

Common and stress-specific molecular biomarkers have been identified through different approaches, and their potential applicability as sub-lethal stress indicators can be verified in the future with *ad hoc* experiments.

Another important aspect of this study is the recognition of the importance of epigenetic variations, specifically DNA methylation changes, as key mechanisms for phenotypic accommodation and adaptive responses to environmental changes in seagrasses.

Tolerance capacity of the species to main current threats of coastal areas, namely the reduction of available light, heat stress, eutrophication and herbivory, is discussed in light of the results obtained from the different experiments.

Keywords: Abiotic and biotic stressors, *Posidonia oceanica*, Light, Heat stress, Eutrophication, Herbivory, Multiple stressors, Temporal variability, Leaf, Shoot-apical meristem, Plagiotropic and orthotropic shoots, Tolerance

“È una umanità che colleziona conoscenza
ma non diffonde la sapienza”

Humanity collects knowledge
But does not spread wisdom

from “Flussi di co-scienza”
Included in the album “Eco Trip”
By Doc Domi & Fabio Musta

List of original papers included in this thesis and author contribution

Chapter II: The first part of the work presented in this chapter has been published previously:

Miriam Ruocco, Lazaro Marín-Guirao, Gabriele Procaccini (2019). Within- and among-leaf variations in photo-physiological functions, gene expression and DNA methylation patterns in the large-sized seagrass *Posidonia oceanica*. Marine Biology 166(3):24.

Author contributions: MR, GP and LMG conceived the experiment. MR, GP and LMG participated in the mesocosm maintenance. MR performed all molecular analyses. MR and LMG performed photo-physiological and morphological analyses. MR conducted the data analysis with the help of LMG and wrote the manuscript. LMG and GP revised the manuscript.

The second part of the chapter has been included in the paper:

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Author contributions: MR, GP and LMG conceived the experiment. MR, GP and LMG participated in the mesocosm maintenance. MR performed all molecular analyses. MR and LMG performed photo-physiological and morphological analyses. MR conducted the data analysis with the help of LMG and wrote the manuscript. LMG and GP revised the manuscript.

Chapter III: The work presented in this chapter will be included in at least two independent publications.

MR, GP and LMG conceived the experiment. MR, GP and LMG participated in the mesocosm maintenance. MR performed all molecular work. MR performed photo-physiological and morphological analyses with the help of LMG. Laura Entrambasaguas performed the transcriptome assembly and differential expression analysis. MR conducted the analysis and interpretation of photo-physiological, morphological and transcriptome data.

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Ruocco M, Musacchia F, Olivé I, Costa MM, Barrote I, Santos R, Sanges R, Procaccini G, Silva J (2017) Genomewide transcriptional reprogramming in the seagrass *Cymodocea nodosa* under experimental ocean acidification. Molecular Ecology 26(16): 4241-4259.

Procaccini G, **Ruocco M**, Marín-Guirao L, Dattolo E, Brunet C, D'Esposito D, Lauritano C, Mazzuca S, Serra IA, Bernardo L, Piro A, Beer S, Björk M, Gullström M, Buapet P, Rasmusson LM, Felisberto P, Gobert S, Runcie JW, Silva J, Olivé I, Costa MM, Barrote I, Santos R (2017) Depth-specific fluctuations of gene expression and protein abundance modulate the photophysiology in the seagrass *Posidonia oceanica*. Scientific Reports 7: 42890.

Olivé I, Silva J, Lauritano C, Costa MM, **Ruocco M**, Procaccini G, Santos R (2017) Linking gene expression to productivity to unravel long-and short-term responses of seagrasses exposed to CO_2 in volcanic vents. Scientific Reports 7: 42278.

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List of acronym and abbreviations

ROS	Reactive Oxygen Species
CSR	Cellular Stress Response
HSP	Heat Shock Protein
PSI	Photosystem I
PSII	Photosystem II
DMT	DNA methyltransferase
HS	Heat Stress
HSR	Heat Stress Response
TF	Transcription Factor
RLC	Rapid Light Curve
NPQ	Non-Photochemical Quenching
F_0	Basal fluorescence
Fm	Maximum fluorescence
Fv	Variable fluorescence
Fv/Fm	Maximum photochemical efficiency of PSII
r-ETR	Relative Electron Transport Rate
ΔF/F'm	Effective quantum yield
Ik	Minimum saturating irradiance
Chl *a*	Chlorophyll *a*
Chl *a*/*b*	Chlorophyll *a*/*b* ratio
Chl *b*	Chlorophyll *b*
PAR	Photosynthetic Active Radiation
RG	Reference Gene
GOI	Genes of Interest
RT-qPCR	Reverse Transcription-quantitative PCR
CT	Threshold Cycle
FC	Fold Change
B	Basal
M	Medium
H	High
LH	Leaf Height
LR	Leaf Rank
5-mC	5-methylcytosine

ETC	Electron Transport Chain
mETC	Mitochondrial Electron Transport Chain
ANOVA	Analysis of Variance
PERMANOVA	Permutational Multivariate Analysis of Variance
PC	Principal Component
PCA	Principal Component Analysis
PCD	Programmed Cell Death
SAM	Shoot-Apical Meristem
LL	Low Light
P	Plagiotropic
O	Orthotropic
MP	SAM of plagiotropic shoot
MO	SAM of orthotropic shoot
ST	Shoot type
DEG	Differentially Expressed Gene
GO	Gene Ontology
BP	Biological Processes
MF	Molecular Functions
CC	Cellular Components
RdDM	RNA-directed DNA methylation
N	Nitrogen
C/N	Carbon/Nitrogen ratio
DIN	Dissolved Inorganic Carbon
Nut	Nutrients
Hrb	Herbivory

Thesis overview

Despite the critical role seagrass meadows play in the equilibrium of costal ecosystems and human livelihoods, the concurrent action of human-caused regional and global impacts, is challenging their persistence. Already large-scale seagrass losses have been reported worldwide, primarily due to reduction of water clarity, heat waves and eutrophication. If from one side, this has led to increased awareness of the need for seagrass protection, from the other side, a more comprehensive knowledge of their tolerance capacity in face of current environmental changes, is imperative for establish proper conservation efforts.

Plant stress response is a very complex trait, heavily dependent upon characteristics of the stressor in question (e.g. intensity and duration) and on intrinsic features of the plant itself (e.g. organ/tissue, developmental stage or genotype). Moreover, the simultaneous action of different stressors increases the variability and uncertainty of the response. This complexity is often ignored, also in seagrass research.

Here, the effect of different abiotic (low light, high temperature and high nutrient levels), and biotic stressors (herbivory) was assessed in the endemic Mediterranean seagrass *Posidonia oceanica*, either individually (light and temperature) or in combination (herbivory and nutrients). Three main experiments have been conducted in the field or in a mesocosm system, spanning from short to long-term exposure, and focusing on plant responses at different levels of organization.

In **Chapter I**, I introduced the concepts of environmental stress and plant stress response, with a special focus on mechanisms and sensors involved, then I provided a framework for the integration of molecular studies in ecological research, and I concluded with a description of seagrass biology and ecology and main threats to seagrass ecosystems. In **Chapter II**, I first investigated the natural variability in molecular (i.e. gene expression and DNA methylation patterns) and photo-physiological functions, within and among seagrass leaves, due to the interplay between developmental and environmental cues. Then, I explored how these gradients of biological properties were modified under an acute warming event (i.e. how the heat stress response can vary within a plant organ). In **Chapter III**, I addressed the response of *P. oceanica* to light limitation in the medium-term. More specifically, the differential transcriptomic response to low light was explored in two different plant organs, i.e. leaf and shoot-apical meristem, and two different shoot types, i.e. plagiotropic and orthotropic. RNA-Seq approach was combined with photo-physiological and morphological assessments. The effect of multiple stressors was addressed in **Chapter IV**, by means of a long-term manipulative field experiment. In particular, I investigated the individual and combined effects of herbivory and variable regime of nutrient loading (i.e. chronic *vs.* pulse)

on the molecular response of *P. oceanica*. Finally, in **Chapter V**, I summarized key findings and concluded by highlighting the importance of considering the complexity of stress response when forecasting the future of seagrass meadows in a global change scenario.

Chapter I – Introduction

Fig. 1.1 A *Posidonia oceanica* meadow. Photo credit: J.M. Ruiz Fernandez

1.1 Stress, stressors and stress response in plants

1.1.1 The stress concept in plants

The ability to constantly sense and adjust to environmental changes is fundamental for all organisms to maintain homeostasis, but it is especially important for plants, as the sessile lifestyle leaves them more exposed to the surrounding environment than animals. The only way to "move" is in fact sexual/asexual reproduction and spatial dispersion of seeds/vegetative fragments.

Plant stress is considered one of the most important topic in plant biology, nevertheless it remains poorly defined, and so far there is no widely accepted, unambiguous definition of this "black box" term (Buchanan et al. 2000; Smith et al. 2010; Taiz and Zeiger 2010).

Selye (1964) and Lichtenthaler (1988) defined a "positive" stress triggered by low levels of a certain stressor as "eustress", and a "negative" stress caused by high levels of a stressor as "distress". The term eustress underlies the adjustments of metabolism that result in a new optimized state under the new environmental conditions, while distress denotes a destructive influence; the balance between eustress and distress determines whether an organism will thrive or die (Jansen and Potters 2017). Selye (1936) and Lichtenthaler (1998) also recognized a different timing of the stress response, with an initial "alarm phase" occurring when an organism is first exposed to a certain stressor, and an "exhaustion phase", after a long-term exposure. The concepts of distress and eustress, somehow paralleled the concepts of "elastic and plastic stress", and thermodynamic state-change, developed by Levitt (1980) and Tsimilli-Michael et al. (1996), respectively. Starting from these theoretical frameworks, Jansen and Potters (2017) elaborated a comprehensive view of the stress concept in plant (Fig. 1.2). Following this view, under mild stress conditions (eustress-prevalent), an imbalance between environmental conditions (eustressor) and physiology (eustress) will occur at the beginning of the exposure, with an initial destabilization of plant functions. Subsequently, an extensive rearrangements of plant metabolism, including gene expression changes, induction of repair/protection responses, morphological and developmental adjustments will occur, leading to "stress acclimation", and ultimately to the optimization of physiological performance under the new environmental conditions (i.e. elastic response). On the contrary, under severe stress conditions (distress-prevalent), an imbalance between environmental conditions (distressor) and physiology (distress) will occurr to such an extent that plant metabolism cannot reach a new optimal state, but rather collapses (i.e. breaking point). This condition is generally associated with high levels of reactive oxygen species (ROS) production, cellular disruption, DNA damage, inactivation of photosynthesis and cell

death. If, however, some repair mechanisms can be activated, a plastic response can take place, with a partial restoration of cellular functions, and/or only local death (e.g. necrosis spots) (Jansen and Potters 2017).

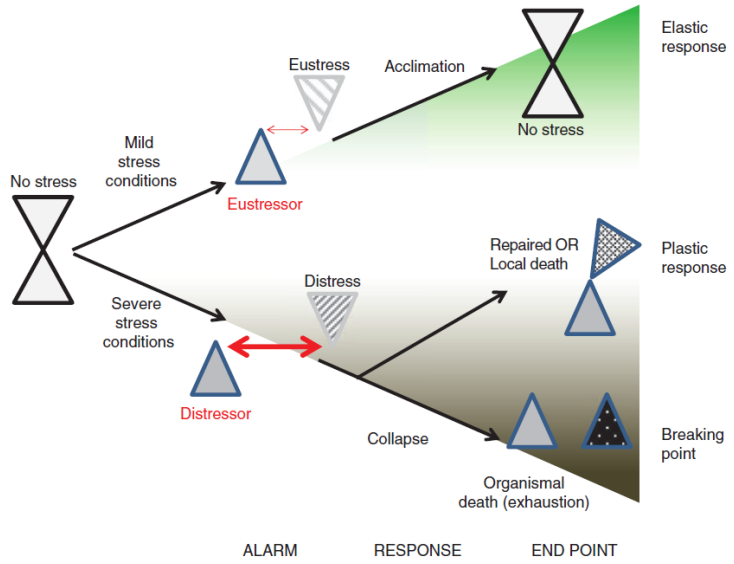

Fig. 1.2 Schematic overview of the plant stress concept. From Jansen and Potters (2017)

In this thesis I adopted the "plant stress" concept as summarized by Jansen and Potters 2017: "*Stress may be defined as a state in which increasing demands made upon a plant lead to an initial destabilization of functions, followed by either normalization and improved tolerance, or permanent damage or death*". All along the text, I used the term "stressor" to indicate the actual environmental factor (physical, chemical or biotic) modified in such a way that it has the capability of causing stress, whereas the term "stress" refers to the plant response.

1.1.2 *Plant stress response*

Plant stress response is a complex mixture of eustress and distress that is affected by several characteristics of the stressor in question and of the plant itself (Buchanan et al. 2015) (Fig. 1.3). Stressor intensity and duration are the most obvious characteristics that influence how a plant responds, and are coupled in the concept of "stress dose". Dose is defined as the magnitude of perturbation times the length of time the stress is applied (Gaspar et al. 2002). The effects of one stressor can be dramatic if applied for a short duration and high intensity, or when it is applied for a long duration at low intensity. Moreover, the number of times the plant is subjected to one stressor, the temporal/spatial variability in the distribution of stress events (e.g. chronic *vs.* pulse), and the combination with other stressors, all may elicit a differential response.

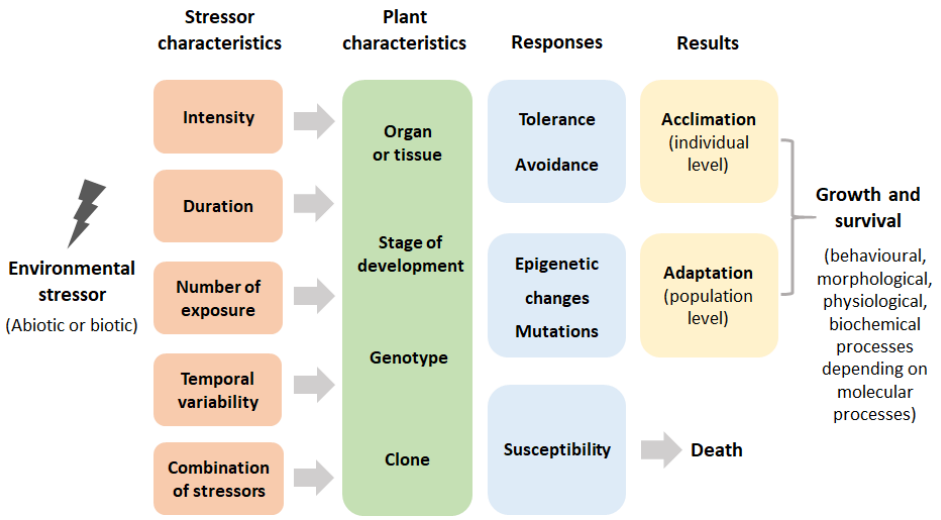

Fig. 1.3 Plant stress responses in correspondence with stressor and plant characteristics. Redrawn from Gaspar et al. (2002)

Intrinsic features of the plant, including organ or tissue type, development stage (e.g. seedling *vs.* adult), and the genetic makeup (i.e. genotype), also influence plant stress response. In addition, within the same genotypes, different individuals (i.e. clones) can exhibit a differential response to the same stressor or stressor combinations (i.e. intraclonal variation) (Buchanan et al. 2015).

Mechanisms that permit plant survival upon exposure to a certain stressor are termed resistance mechanisms and are grouped in two general categories: avoidance and tolerance mechanisms[1]. Avoidance mechanisms prevent plant exposure to the stressor through a drastic reduction of metabolic activities, resulting in a dormant state; tolerance mechanisms enable plants to withstand the stressor, maintaing metabolic activity at high (or moderate) level (Gaspar et al. 2002; Buchanan et al. 2015). Some tolerance traits are constitutive and thus expressed wheter the stressor is present or not. These traits are typically genetically determined and constitute evolutionary improvements (i.e. adaptations) that enhance the fitness of populations. Other tolerance traits result from acclimation processes, and are induced following the stressful event. Generally, plants can exhibit several tolerance and avoidance mechanisms, or a combination of both (Buchanan et al. 2015).

When addressing plant stress response, the distinction between adaptation and acclimation processes becomes particularly relevant. Adaptation occurs at the genetic level in plant populations over many generations, via microevolutionary processes; acclimation is instead

[1] It must be noted that in case of biotic stressors (e.g. herbivore/pathogen attack) the terms "resistance" and "tolerance" are used in the literature with a slightly different meaning, as discussed in details in the Chapter IV, here I refer to resistance and tolerance as general terms indicating plant strategies in response to any kind of stressors.

a phenotypic response of the individual plant under different combinations of environmental settings (Gaspar et al. 2002). Following this concept, phenotypic plasticity can be considered as the "amount of acclimation" that is possible within a genotype (Nilsen and Orcutt 1996). Adaptation and acclimation occur through a combination of morphological, physiological and biochemical processes, which are primarily determined at molecular level, and permit plant survival under a wide range of environmental conditions.

Ultimately, environmental stressors represent one the major forces shaping plant structure and function. They should not be automatically associated with detrimental effects, rather, up to certain extent, they can represent on "opportunity" for plants and can confer new adaptive advantages.

1.1.3 *Multiple stressors*

As stated before (see 1.1.2.), plant response to one stressor can be modified by the simultaneous or subsequent exposure to another stressor, therefore is it necessary to provide a theoretical framework of response to multiple stressors. This is particularly important as, in natural settings, organisms rarely encounter purely individual stressors and more often need to deal with more than one stressor at a time (Todgham and Stillman 2013).

Conceptually, organisms subjected to multiple stressors can exhibit one of three types of responses: additive, antagonistic, or synergistic (Fig. 1.4) (Crain et al. 2008; Todgham and Stillman 2013). If there is no interaction, the combined effect of two stressors is said to be additive, as it equals the sum of the effects of each stressor in isolation. Conversely, interactions between stressors can be synergistic or antagonistic; an antagonistic effect arises when the combined effect is less than the expected additive effect in isolation, whereas a synergistic effect occurs when the combined effect of multiple stressors is greater than the expected additive effect of the individual stressors (Todgham and Stillman 2013).

Despite the common use of these definitions, many complications arise when labelling the different ways in which multiple stressors can interact in a realistic context. For example, while the identification of a synergism or antagonism is generally straightforward when both stressors operate in the same direction on the biological response of interest, when they operate in the opposite direction the definition of synergism becomes paradoxical (Piggott et al. 2015). Similarly, when two stressors operating in the same direction create a cumulative effect completely opposite to what would have been predicted (e.g. they synergistically mitigate or inhibit their individual effects even more than under control conditions) the definition of synergism becomes misleading. This led to the introduction of new classes of "positive synergism" and "mitigating synergism" (Piggott et al. 2015). Other

complications become evident when considering the response of certain variables for which the additive null model (i.e. the sum of the stressor effects when acting in isolation) underlying the interaction type is not applicable (e.g. in the case of mortality estimation, individuals killed by one stressor cannot be killed by the other stressor) (Côté et al. 2016). The prediction of multiple stressor effects thus remains a very complex task. Moreover, most studies measure the effect of multiple stressors only in terms of their impacts on organisms' abundance, survival, growth rate, biomass, etc. (i.e. phenomenological studies) without examining the underlying causes (e.g. physiological or molecular) at the individual level (i.e. mechanistic studies) (Griffen et al. 2016). This limits their predictive power, the possibility to extend results beyond the specific context, to different species or different environmental conditions, and to identify common pathways of multiple-stressor response (Griffen et al. 2016).

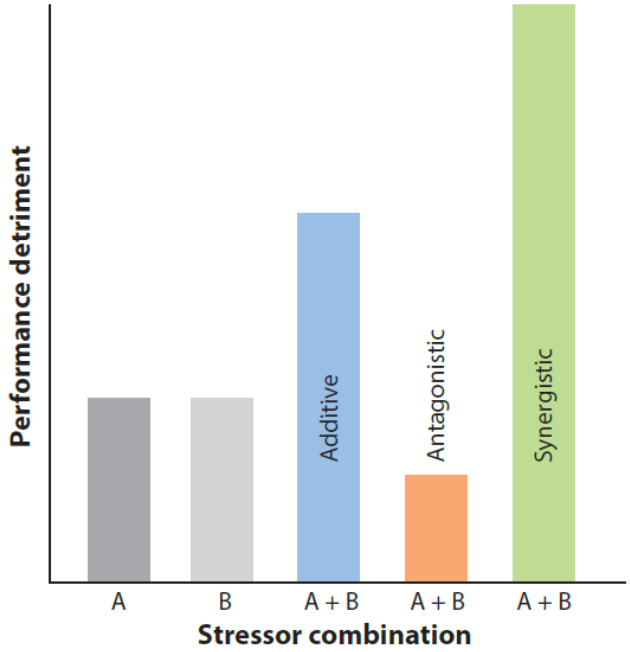

Fig. 1.4 Conceptual diagram of possible effects of two stressors on physiological performance. From Gunderson et al. (2016)

1.1.4 Tuning plant gene expression in response to stress

The modulation of gene expression has a central role in plant plasticity and adaptation to environmental changes (DeWitt et al. 1998), since physiological machinery and metabolic pathways are coordinated at the genetic level by an array of regulatory genes, which are affected by environmental stimuli (Pigliucci 1996).

A stress response is generally initiated when a plant recognizes a stress at the cellular level. Signal-transduction pathways are then activated and translate extracellular signals into

specific intracellular responses. Ultimately, different gene expression programs are launched and integrated into a response at the whole plant level (Rao et al. 2006).

Studies on stress-induced changes in gene and protein expression have revealed the presence of a phylogenetically conserved core cellular stress response (CSR), that is triggered by all taxa in response to a wide range of environmental perturbations (Kültz 2003; Kültz 2005). At the most basic level, cells respond to all types of stressors by activating a set of genes and pathways aimed at stabilizing macromolecule structure and function, and at conserving metabolic energy for long-term cellular homeostasis response. Main CSR mechanisms include: (1) cell cycle checkpoint control leading to growth arrest; (2) induction of molecular chaperones (e.g. heat shock proteins); (3) activation of systems for nucleic acid and chromatin stabilization and repair; (4) removal of macromolecular debris (e.g. through the ubiquitin/proteasome pathway); (5) activation of programmed cell death when the severity of stressor exceeds cell tolerance limits (Kültz 2003; Kültz 2005). All these mechanisms are interconnected via common stress signaling networks, and are generally activated at the expense of other cellular functions, meaning that CSR is only a transient response, giving cell the time for re-establishing a long-term cellular homeostasis response, which is more specific for the stressor in question (Kültz 2003).

Genes and pathways associated to the CSR represent the minimal stress transcriptome and proteome conserved in all organisms. However, a myriad of other genes have a specific role in plant stress response, as demonstrated by the application of genome-wide approaches which are now providing a global view on gene expression responses to many different abiotic and biotic stressors (Mosa et al. 2017). Plants exhibit “shared” and “unique” stress responses, where shared responses refer to the molecular responses which are common to different stressors and unique responses are the ones specific to individual stressors (Pandey et al. 2015). Notably, the identification of shared and unique mechanisms constitutes the basis for identifying biomarkers of plant stress tolerance (Kosová et al. 2014; Pandey et al. 2015). An overview of major shared plant responses to external stressors is depicted in Fig. 1.5. Generally, both abiotic and biotic stressors induce profound alterations in plant energy metabolism, since stress acclimation requires high energy costs (Kosová et al. 2011; Kosová et al. 2014; Kosová et al. 2015). Specifically, an increase in relative abundance of transcripts and proteins involved in carbohydrate metabolism such as glycolysis, tricarboxylic acid (TCA) cycle, and components of mitochondrial respiratory chain, including ATP synthase, is observed (Kosová et al. 2014). Regarding the photosynthetic process, an increase or decrease in several enzymes/structural components involved in photochemical reactions and carbon fixation (e.g. Calvin cycle) is detected depending on the severity of the stressor (see

1.1.5 for further details). At the same time, the increase in internal energy demand under stress conditions, parallels the decline in energy-rich compounds biosynthesis (e.g. starch) and suppression of related enzymes, and storage proteins (Kosová et al. 2014). Acclimation to several stressors triggers enhanced protein metabolism, either biosynthesis and degradation, as demonstrated by changes in the expression levels of several components of the translation machinery (e.g. ribosomal proteins, translation initiation and elongation factors etc.), as well as members of ubiquitin pathway and proteasome subunits (Kosová et al. 2014). As common in the CSR, an increase in the abundance of several transcripts and proteins functioning as chaperones or involved in other protective functions is always reported (e.g. HSPs, protein disulfide isomerases), together with the activation of ROS scavenging enzymes (e.g. catalase, ascorbate peroxidase, and superoxide dismutase) (Das and Roychoudhury 2014). It is important to note the ROS (including free radicals like $O^{\bullet-}_2$, $OH^{\bullet}$ and non-radicals like H_2O_2 and $^1O_2$) produced under several stress conditions in plant chloroplast and mitochondria, play a double role: from one side, they induce extensive oxidative damages to several cellular components (e.g. DNA, pigments, proteins, lipids etc.), from the other side they are integral to plant stress signaling, acting as fundamental secondary messengers (Choudhury et al. 2013; Das and Roychoudhury 2014). The balance between ROS production and elimination is influenced by the severity of stress, and ultimately determines plant tolerance or susceptibility to a certain stressor (Liebthal and Dietz 2017). Several other biosynthetic pathways such as S-adenosylmethionine metabolism, which provides methyl groups in regulation of DNA heterochromatin formation and gene expression, lignin metabolism, as well as fundamental enzymes involved in nitrogen assimilation (e.g. glutamine synthetase), ion transporters and protective proteins (e.g. LEA and PR superfamily), are regulated under several stressors, at transcript and/or protein level (Kosová et al. 2014).

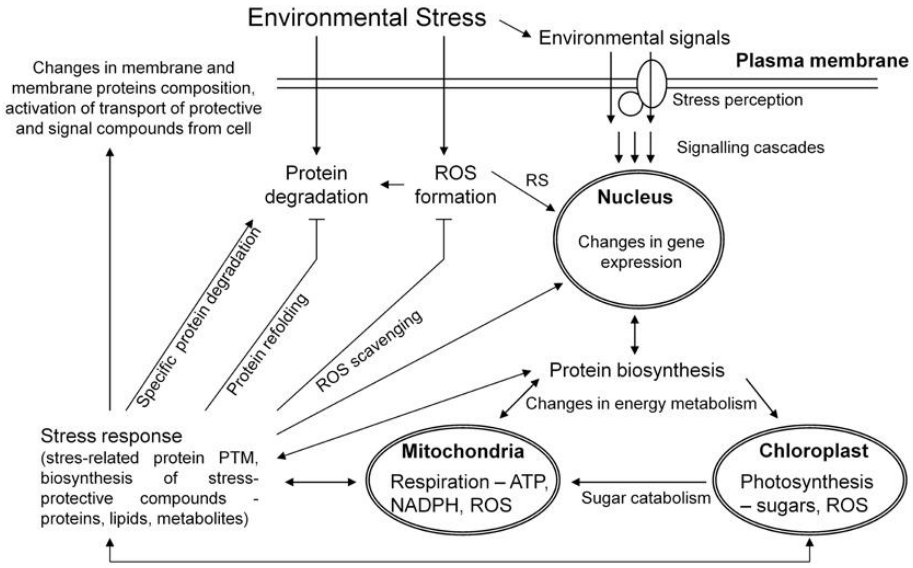

Fig. 1.5 Schematic overview of plant responses to environmental stressors triggering signaling cascades, changes in gene expression, activation of protein biosynthesis/degradation, and changes in energy metabolism resulting in ROS induced signaling. From Kosová et al. (2014)

1.1.5 *Photosynthesis as a global stress sensor in plants*

As discussed in 1.1.4, environmental stressors cause a perturbation in energy homeostasis; therefore, plant stress acclimation and subsequent tolerance capacity are based on the re-establishment of cellular energy balance. In this context, photosynthesis, the fundamental energy-producing process in plants, plays a central role as stress sensor, where it modulates energy signalling and balance (Biswal et al. 2011). In recent years, it has been clearly demonstrated that the functional state of the photosynthetic process has a direct impact on the expression of genes encoding its own constituents via several redox-reactive regulatory molecules (Pfannschmidt 2003; Pfannschmidt et al. 2009; Pfalz et al. 2012; Queval and Foyer 2012). All the genes showing redox-regulated expression are indeed related to photosynthesis and are encoded either by the chloroplast or nuclear genome (Pfannschmidt 2003). Thus, chloroplast redox signals directly help plants to acclimate to changing environmental conditions where they modulate expression levels of photosynthetic components at transcriptional and post-transcriptional level.

In Fig. 1.6 are depicted major photosynthetic sensors, signals arising from stress-induced changes in these sensors, and ultimate short and long-term plant responses. Among all photosynthetic components that are known as stress targets, photosystem I and II (PSI and PSII) and the Rubisco enzyme act as primary sensors in leaf chloroplasts. Specifically, PSII is considered more susceptible than PSI, the most sensitive part being the metal centre of the oxygen evolving complex (Biswal et al. 2011). Disturbance of sensors following stress events generate signals like energy imbalance, redox changes associated with electron

transport system (e.g. plastoquinone pool and thioredoxin), production of ROS and changes in the cellular sugar level (Biswal et al. 2011). These changes result in photochemical, metabolic and molecular reprogramming, through several signal transduction pathways. The leaf can exhibit short-term acclimation mechanisms like state transition with a change in PSII absorption cross-section, alteration in PSII:PSI stoichiometry and dissipation of excess energy as heat through non-photochemical quenching (NPQ) of chlorophyll excited state (Biswal et al. 2011). Extensive nuclear and chloroplast gene-expression changes, regulated by stress-induced alteration in redox status of electron transport components and in the level of cellular sugars, are associated with long-term stress acclimation capacity (Biswal et al. 2011).

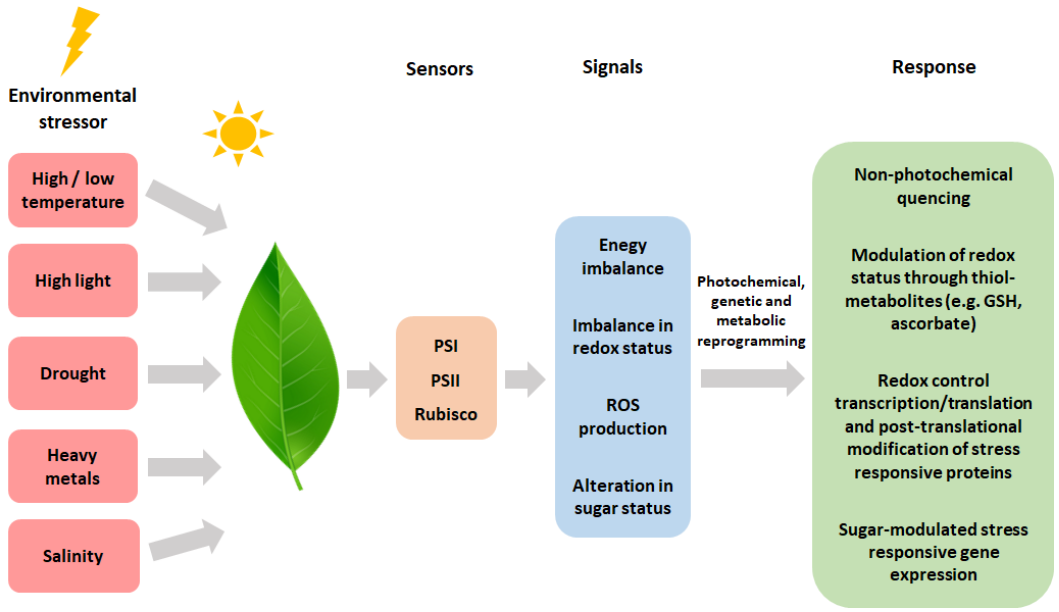

Fig. 1.6 Scheme depicting major events associated with chloroplast-specific stress sensing mechanisms and responses in plants experiencing various environmental stressors. Redrawn from Biswal et al. (2011)

1.1.6 *Epigenetic mechanisms of plant stress response*

Stress acclimation is defined as "the induction of reversible, non-heritable, physiological or biochemical responses that lead to increased tolerance" (Jansen and Potters 2017). However, the presence of reversible epigenetic changes modulating plant response to environmental stressors that can be inherited across generations, challenges this definition.

Epigenetic modifications of genomes include all changes in and around DNA, which do not alter DNA sequence itself; they include chemical modifications of the DNA (e.g. methylation) and its associated proteins (e.g. post-translation histone modifications), or involve RNA molecules (e.g. gene silencing by non-coding RNAs) (Allis and Jenuwein 2016). These multiple epigenetic processes are critical to regulate the condensation state of

chromatin; they modulate DNA accessibility by RNA polymerase, transcription factors and DNA binding molecules and consequently directly affect its expression both during development and in response to environmental stressors (Allis and Jenuwein 2016).

A number of studies have shown that epigenetic modifications play a key role in regulating plant gene expression under stress conditions, the bulk of epigenetic studies being essentially focused on DNA methylation (the addition of methyl groups to cytosine nucleotides) and chemical changes of histone proteins (Chinnusamy and Zhu 2009; Mirouze and Paszkowski 2011; Kumar 2018). Notably, while most epigenetic stress-induced modifications are reset once the stress is relieved, some of them may be stable and carried forward as 'stress memory', that is inherited across mitotic or even meiotic cell divisions (Chinnusamy and Zhu 2009). This has been clearly demonstrated in plants (Verhoeven et al. 2010; Latzel et al. 2012), however the stability of the epigenetic transmission across generations is not well characterized (Herman et al. 2014). Above all, epigenetic changes may play a major role not only in plant stress acclimation, but also in long-lasting plant adaptation strategies to environmental changes.

Especially clonal plants may benefit from epigenetic acclimation (and its adaptive potential) as an alternative to the slower mechanisms of adaptation through natural selection (Douhovnikoff and Dodd 2015; Dodd and Douhovnikoff 2016). The lack of recombination as a source of genetic variation, accompanied by limited dispersal capacity could indeed compromise their capacity to migrate or evolve fast enough to cope with environmental challenges. Recent works suggest that ecological advantages of the clonal growth strategy, together with epigenetically regulated plasticity, can explain in part the success of clonality (clonal plants represent around 40 % of planet's flora) and could be a mechanism that will buffer them against current and future rapid climate changes (Douhovnikoff and Dodd 2015; Dodd and Douhovnikoff 2016). Yet, epigenetic responses seem to favor long-living organisms since they can build through time. For example, the first individuals exposed to new local settings may not be optimally acclimated, but over a number of ramet generations, the new modules could progressively acclimate as more epigenetic responses develop, increasing their fitness over time (Douhovnikoff and Dodd 2015).

1.2 Integration of gene expression, physiology and ecology in the study of environmental stress in marine organisms

Several recent reviews have underscored the advantages of using genomic technologies (including the study of gene-expression variations) in ecologically relevant studies (Jackson et al. 2002; Thomas and Klaper 2004). They conclude that eco-genomics approaches are essential for understanding not only the acclimation responses and adaptive potential of organisms to environmental changes, but also the distribution and interactions of organisms over time and in space (Hofmann et al. 2005; Somero 2010; Evans and Hofmann 2012) (Fig. 1.7).

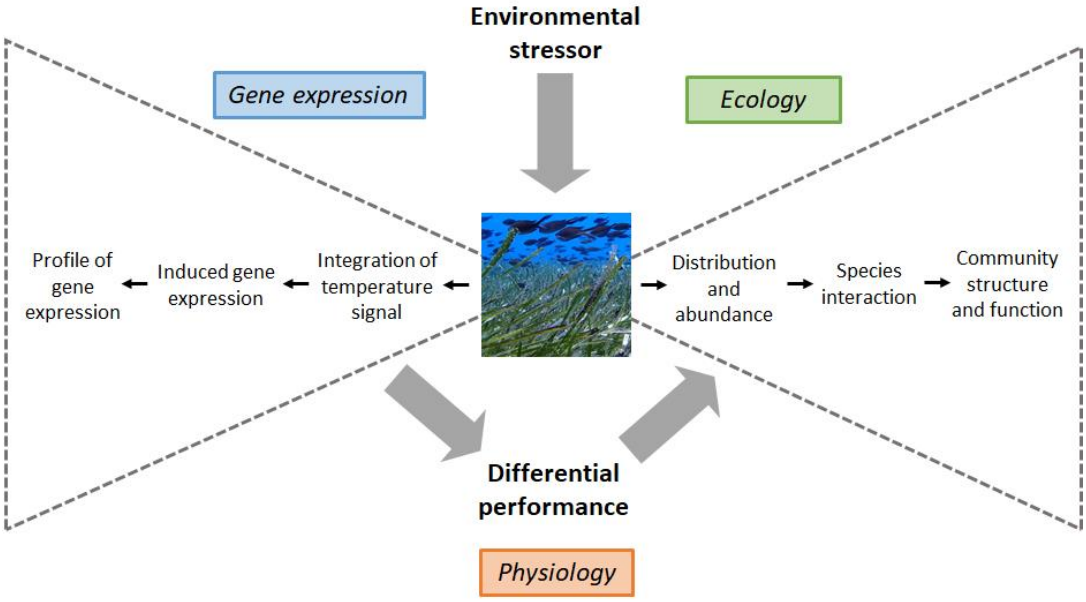

Fig. 1.7 Integration of molecular biology, physiology and ecology in the study of environmental change. Redrawn from Hofmann et al. (2005)

Especially coastal marine species are being challenged from multiple (and interacting) threats related to anthropogenic activities, localized mostly along nearshore areas. As such, major deviations from the prevailing abiotic and biotic conditions that dominated their evolutionary history are occurring, challenging marine biodiversity and ecosystem function (Harley et al. 2006; Jackson 2008).

The possibility to examine shifts in expression levels of several thousands of genes simultaneously e.g. through transcriptomic approaches, can provide a comprehensive view of molecular changes that accompany species alterations in physiological state (Gracey 2007; Evans and Hofmann 2012). Differential physiological performance will then reflect into a differential organismal distribution and abundance that will ultimately modify community structure and function (Fig. 1.7). This is especially true when examining the

effects of environmental stressors on ecologically relevant species (e.g. keystone species or ecosystem engineers), the loss of which will directly affect associated organisms (Hofmann et al. 2005). In an ecological context, the concept of environmental stressor for a certain individual/species (as defined in 1.1.1) can be extended to community composition and ecosystem functioning.

If full transcriptomic analyses can provide important insight into overall changes in gene expression, target gene-expression approach (e.g. quantitative real-time PCR analysis) can yield detailed dynamics of transcriptomic changes for a limited number of genes of particular interest, that can be directly linked with key physiological processes (Zheng and Dicke 2008). More importantly, such genes can be used as molecular biomarkers, which provide the earliest possible evidence of stress, far exceeding that of morphological and physiological indicators (Macreadie et al. 2014). Changes in the expression of genes related to the CSR ranked along increasing severity levels, represent a means to establish sub-lethal stress markers, and tolerance thresholds for physiological functions, before mortality takes over (Evans and Hofmann 2012; Traboni et al. 2018). For example, the induction of molecular chaperones would indicate stress levels that temporarily compromise protein function, while the simultaneous expression of genes involved in protein folding, proteolysis and cell cycle regulation would occur closest to organism-tolerance limits (Evans and Hofmann 2012).

Similarly, a new generation of biomarkers of marine pollution based on dynamic epigenetic modifications has been proposed, as already established for terrestrial model organisms. Epigenetic marks indeed constitute dynamic and potentially reversible modifications, as such they represent outstanding candidates for developing fast and sensible environmental biomonitoring programs in marine ecosystems (Suarez-Ulloa et al. 2015).

Ultimately, a major challenge is to integrate approaches that address different levels of biological organization, from subcellular mechanisms to physiological functions and ecological communities.

1.3 Seagrass ecosystems under multiple stressors

1.3.1 Seagrass biology, ecology and evolution

Seagrasses are a polyphyletic group of monocotyledonous angiosperms that have adapted to a completely submerged lifestyle in marine waters (Den Hartog 1970). As the "whales" of the plant kingdom, they returned back from the land to the sea, preserving some features of their terrestrial counterparts. The recolonization of marine habitats occurred at least three times independently through parallel evolution from a common aquatic-freshwater ancestor of terrestrial origin, and appears to be evolutionary unique (Les et al. 1997; Waycott et al. 2006). Seagrasses belong to the monocot order of Alismatales and comprise four families, namely *Posidoniaceae*, *Zosteraceae*, *Cymodoceae*, and *Hydrocharitaceae*, which have originated in the Creataceous period (Green and Short 2003). Although seagrasses exhibit a low taxonomic diversity (approximately 60-70 recognised species), they are widely distributed along temperate and tropical coastlines of all continents except Antarctica. All families also occur in the Mediterranean bioregion (Green and Short 2003).

Most seagrasses exhibit a combination of clonal growth and sexual reproduction. The bulk of seagrass bed expansion generally occurs through vegetative fragmentation, although seed production is important for maintaining genetic diversity within populations and as agents of long-distance dispersal (Waycott et al. 2006). Clonality results in a hierarchy of different organizational levels. The basal level is the "ramet", the potentially independent individual, typically consisting of a leaf bundle, a piece of rhizome, and a root bundle (Waycott et al. 2006). Several ramets can form physiologically integrated clusters (the second organization level), that may comprise up to several hundreds of individuals in some genus (e.g. *Posidonia*). The sexual individual, i.e. "genet", comprises all ramets or ramet clusters originated from the same zygote (Waycott et al. 2006).

Living submerged poses many challenges requiring morphological, physiological, and biochemical adaptations (Den Hartog 1970). The most essential differences in respect to terrestrial angiosperms are i) the lack of stomata, ii) extremely thin cuticle, iii) epidermis as the main photosynthetic tissue, and iv) reduced water-conductive elements, while other features can vary among seagrass species (Kuo and Den Hartog 2007). Seagrasses complete their entire life cycle in the aquatic medium, including flowering, pollen transport and seed germination (Kuo and Den Hartog 2007). At the physiological level, the photosynthetic apparatus needs to be modulated to accommodate changes in light intensity and quality through the water column. Accordingly, seagrasses have one of the highest light requirements among angiosperms (Dennison et al. 1993). Factors contributing to these high

light requirements are the anoxic sediments to which seagrasses are rooted and the need to support large amounts of non-photosynthetic tissue (Terrados et al. 1999). Yet, seagrasses rely on carbonic acid and bicarbonate instead of CO_2 for photosynthesis (Beer and Rehnberg 1997; Invers et al. 1999; Larkum et al. 2017), due to the reduced availability of CO_2 in seawater, and have evolved special physiological mechanisms to deal with high salt levels and short-term salinity fluctuations in coastal and estuarine systems (Barbour 1970; Walker and McComb 1990). At molecular level, seagrass adaptations to the marine lifestyle have been achieved through specific genomic losses and gains (Golicz et al. 2015; Olsen et al. 2016) and adaptive changes in sets of genes associated with central biological pathways, such as translation, photosynthesis, and glycolysis (Wissler et al. 2011).

Because of the key ecological services they provide to the coastal zone and human livelihoods (Cullen-Unsworth et al. 2014; Nordlund et al. 2017), seagrass-based ecosystems rank amongst the most valued on earth, surpassing the economic value of coral reefs and tropical rainforests (Costanza et al. 1997; Barbier et al. 2010). The habitat complexity within seagrass meadows enhances the diversity and abundance of associated species from all trophic levels (Hughes et al. 2008; McCloskey and Unsworth 2015). They serve as feeding and nursery areas for many commercially and recreationally important species of fishes, mollusks, and crustaceans, thus supporting global fishery (Nordlund et al. 2018; Unsworth et al. 2018). As well, seagrass meadows oxygenate water, stabilise sediments, provide shoreline protection from erosion (Koch et al. 2009), and are natural hotspots for carbon sequestration (Kennedy et al. 2010; Fourqurean et al. 2012; Tanaya et al. 2018) and nutrient cycling. In particular, the importance of seagrass as long-term "blue carbon" sink is disproportionally greater compared with terrestrial ecosystems, thus they are expected to contribute greatly to the mitigation of anthropogenic CO_2 emissions, and ultimately global warming (Mcleod et al. 2011). Recently, the ability of seagrass meadows to reduce exposure to bacterial pathogens capable of causing disease in humans and marine organisms, has been demonstrated (Lamb et al. 2017).

Posidonia oceanica

Posidonia oceanica (L.) Delile (Fig. 1.8) is the most wide-spread species in the Mediterranean, and is also endemic to this sea (Short et al. 2007). It is a monoecious, hermaphroditic species with irregular flowering and fruiting (Jahnke et al. 2015), characterized by extremely slow rhizome elongation rates (1–10 cm per year), a high dispersal potential of reproductive structures and drifting vegetative fragments, and long persistence of genotypes (Procaccini et al. 2003; Arnaud-Haond et al. 2012). *P. oceanica*

grows according to a phalanx strategy (Migliaccio et al. 2005), that is the connections between ramets have few and/or short internodes, resulting in closely packed ramets (Ye et al. 2006). This growth form is typical of late successional ecological stages and relatively homogeneous/less disturbed habitats, and enables clonal plants to tolerate more stressful conditions, make better use of locally available resources and out-compete other species in favorable microsites (Ye et al. 2006). *P. oceanica* forms extensive monospecific meadows on rocky and sandy bottoms, which are considered one of the climax communities in the Mediterranean coastal area (Procaccini et al. 2003). The depth distribution range of *P. oceanica* populations is wide and span from less than 1 meter down to 45 meters. Shallow and deep meadow stands show signs of local adaptation, with a pronounced genetic structure and reduced gene flow (Migliaccio et al. 2005; Jahnke et al. 2018), and significant divergence in gene-expression patterns related to light and temperature regime shifts (Dattolo et al. 2013; Dattolo et al. 2014; Dattolo et al. 2017; Procaccini et al. 2017; Jahnke et al. 2018).

Fig. 1.8 *P. oceanica* and its associated biodiversity. Photo credit: M. Ruocco and G. Procaccini

P. oceanica is a large-sized seagrass with extremely high biomass and productivity. Leaf bundles consist of 5 to 10 leaves, with a width of ca. 1 cm and a length that can reach up to 120 cm (Larkum et al. 2006). Shoots are sustained by rhizomes growing either vertically (orthotropic rhizomes), or horizontally (plagiotropic rhizomes), the latter are more typical of areas undergoing colonization. The progressive silting and the alternation of the two types of rhizome growth result in the formation of the "matte", a typical terraced structure consisting of the intertwining of various strata of rhizomes, roots, and sediment (Larkum et al. 2006). These structures can persist *in situ* with little alteration for millennia, arising for meters above the sediment level (Mateo and Romero 1997; Procaccini et al. 2003), and may be considered a form of bioconstruction (Bianchi 2001). Accordingly, *P. oceanica* meadows possess the largest documented pools of C_{org} stores of any living seagrasses (Fourqurean et al. 2012). The economic value associated to ecosystem services provided by *P. oceanica* and their contribution to human well-being have been recently evaluated through different approaches (Vassallo et al. 2013; Campagne et al. 2015). The diagram in Fig. 1.9 by Vassallo et al. (2013) depict the *P. oceanica* system and its main services: nursery role, sediment retention and hydrodynamics attenuation, primary production and oxygen release. Such services are maintained by sun, nutrients, carbon dioxide and sediment, that are the main inputs to the system, whereas the outputs arising from these services are fish biomass, shore protection, water oxygenation and plant biomass. According to Vassallo et al. (2013) the monetary value of *P. oceanica* is nearly two orders of magnitude greater of that proposed by Costanza et al. (1997) for seagrasses in general, and the service contributing most to this estimation is the sediment retention by the meadow.

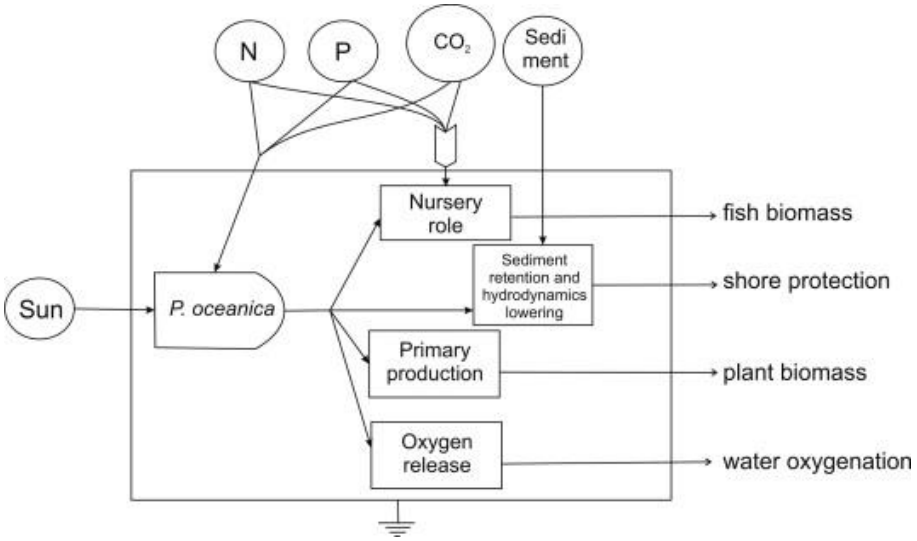

Fig. 1.9 System diagram of *P. oceanica* services. From Vassallo et al. (2013)

1.3.2 Threats to seagrass ecosystems

Despite their ecological value, seagrass meadows are declining worldwide (Orth et al. 2006; Marbà et al. 2014), due to a number of local threats associated to human activities along the coastline (urban/port infrastructure development, trawling, urban/industrial runoff, aquaculture, recreational boat damage, agricultural runoff, dredging, invasive species and desalination plants; Grech et al. 2011, 2012), as well as global climate changes, including increase of sea level and harmful UV radiation in shallow waters, variation of salinity, raising mean and extremes of sea temperature, drop in pH and a host of secondary changes (Short and Neckles 1999; Orth et al. 2006; Waycott et al. 2009; Hoegh-Guldberg and Bruno 2010; Gruber 2011; Koch et al. 2013). The concurrent action of climate and non-climate stressors amplifies the negative effects on seagrass meadows, and this in turn affects associated organisms and communities (Orth et al. 2006).

At least 1.5% of seagrass meadows is lost every year and nearly 29% of their areal extent has disappeared since 1879, implying that 1/3 of goods and services they provide has been already lost (Waycott et al. 2009). Currently, ten seagrass species are considered at elevated risk of extinction, and three species are qualified as endangered (Short et al. 2011).

Particularly, the conservation of *P. oceanica* meadows has become a key objective on actual European environmental and water policies. *P. oceanica* is protected at the European level, as a priority habitat (Annex I Habitats Directive/NATURA 2000 habitat - code: 1120; *Posidonia* beds) and as a species (Bern Convention, Annex 1), and is under specific legal protection actions in several European countries. *Posidonia* is also used as a bio-indicator species for good environmental/ecological status in many national and international monitoring programs aiming at improving quality of coastal waters and marine environments such as Water Framework Directive and Marine Strategy Framework Directive.

The most recent study assessing *P. oceanica* distribution and trajectories of change (Telesca et al. 2015), estimated that meadow loss amounted to 34% in the last 50 years. *P. oceanica* is a slow-growing species (see 1.3.1), and its regression is considered irreversible at human scale, while other seagrasses (e.g. fast-growing species) can rapidly recover after stress events. Yet, the expansion of some seagrass (e.g. *Cymodocea spp.*) cannot counterbalance, in terms of ecosystem services, the decline of *P. oceanica* meadows (Boudouresque et al. 2009). The management of direct impacts, such as trawling, anchoring or dredging, can help the recovery of *Posidonia*, although this can take an extremely long time (Badalamenti et al. 2011; Fraschetti et al. 2013), whereas transplantation efforts on a large scale have been often unsuccessful (Sánchez-Lizaso et al. 2009).

Recently, major seagrass mass mortality events have been ascribed to ocean warming and particularly to marine heat waves (Marbà and Duarte 2010; Fraser et al. 2014; Thomson et al. 2014; Arias-Ortiz et al. 2018). In the Western Mediterranean, the temperature increase following a heat wave led to significant *in situ* shoot mortality and overall decline in *P. oceanica* across the Balearic Islands (Marbà and Duarte 2010). In light of these findings, Jordà et al. (2012) foresaw future trajectories of *P. oceanica* meadows in an ocean warming scenario, where the species will retain approximately 10% of the present density by 2049. However, a recent mesocosm experiment simulating summer heatwaves more severe than those reported above, revealed a tolerance capacity of *P. oceanica* higher than expected, with no induced mortality and a complete recovery of photo-physiology, growth and carbon/energy content after the stress cessation (Marìn-Guirao et al. 2018). Although some caution should be taken due to the limitations imposed by the mesocosms approach, this rises doubt on the heat-induced extinction forecasted for the species by the middle of the 21th century by Jordà et al. (2012).

1.3.3 *Molecular stress response studies in seagrasses: state of the art*

Gene expression studies are growing also in non-model species, including seagrasses (Procaccini et al. 2007). In recent years, several experiments have been carried out using both target-genes (e.g. RT-qPCR) and "omic" (e.g. cDNA libraries, 454 pyrosequencing, Illumina RNA-seq) approaches, under controlled conditions in mesocosm systems or in the field. These studies, besides shading first light on plastic and adaptive responses of seagrasses to environmental stressors, allowed the development of first transcriptomic resources (Franssen et al. 2011; D'Esposito et al. 2016; Entrambasaguas et al. 2017; Marín-Guirao et al. 2017; Ruocco et al. 2017), which represent an important foundation for future studies in these species. However, it should be noted that abovementioned studies are limited to a very small number of species, mainly *Zostera* spp. and more recently *P. oceanica* and *C. nodosa*, while for many other seagrass species molecular studies and resources are completely absent. In the following sections, I will discuss results of gene-expression studies conducted in seagrasses focusing on plant responses to single environmental stressors.

High / low light

First insights have been given into molecular mechanisms underpinning acclimation /adaptation strategies to different light regimes in *P. oceanica* populations extending along bathymetric gradients (Dattolo et al. 2013; Dattolo et al. 2014; Procaccini et al. 2017).

Specifically, *Posidonia* seems to be better adapted to low-light conditions experienced by the species at higher depths, while it has to activate specific photoprotective mechanisms to cope with high light in shallow stands, as shown by the strong up-regulation of photosynthesis and photoprotection-related genes and antioxidant enzymes (Dattolo et al. 2014). Moreover, when analyzing the daily oscillatory patterns of gene expression, a response asynchrony between shallow and deep-growing plants is observed, and this reflects the diel phases of photo-physiological and respiratory responses (Procaccini et al. 2017). Mesocosm-based experiments confirmed the differences existing between stands growing at different depths, and showed that *P. oceanica* plants conserve memory of their original conditions, when exposed (i.e. transplanted) to contrasting light regimes (Dattolo et al. 2017), providing evidence for local adaptation. Recent genome-wide transcriptome analysis conducted between contrasting depth in *P. oceanica*, identified flavonoid and lignin biosynthesis-coding genes, as well as genes involved in cell-wall loosening, as the most divergent, proving evidence that the production of secondary metabolites and cell wall remodeling are among the main pathways involved in the acclimation of shallow and deep population (Jahnke et al. 2018).

High CO_2 / low pH

Few studies so far have addressed the molecular responses of seagrasses to high CO_2/low pH conditions, since the projected increase in CO_2 level is generally considered beneficial for CO_2-limited organisms like seagrasses (Hall-Spencer et al. 2008). In a first study, Lauritano et al. (2015) addressed the expression of 35 stress-related genes in *P. oceanica* growing under nearby CO_2 vents in two islands in the Mediterranenan: Ischia and Panarea. An up-regulation of genes involved in the free-radical detoxification response was found only at the Panarea site, suggesting that here *P. oceanica* faces stressors that result in ROS production. In addition, HSPs were also activated in *P. oceanica* at Panarea and not at Ischia, suggesting the presence of environmental and/or evolutionary differences between the two volcanic spots (Lauritano et al. 2015). The long- and short-term (i.e. transplant) responses of the seagrass *Cymodocea nodosa* under high-CO_2 level near the volcanic vents of Vulcano Island were assessed by Olivè et al. (2017). In contrast with expectations, a consistent long- and short-term pattern of gene expression down-regulation and net plant productivity (NPP) decrease in plants incubated in water from the CO_2 vents was observed. Conversely, when plants from the vent site were incubated with control water an up-regulation of most genes and an increase in NPP was observed (Olivè et al. 2017). The first RNA-Sequencing to explore seagrass response to ocean acidification (OA) in controlled conditions has been

performed in *C. nodosa* (Ruocco et al. 2017). This study suggests that the increase in the respiratory activity, driven by a greater abundance of transcripts encoding enzymes throughout the respiratory pathways, is a central metabolic mechanism to cope with OA in seagrasses, and supports an augmented energy demand for protein turnover and ion/pH homeostasis maintenance. OA further modifies *C. nodosa* secondary metabolism, inducing the transcription of enzymes related to carbon-based-secondary compounds (Ruocco et al. 2017).

Heat stress

Several intra and inter-specific differences in heat stress response were identified through common garden experiments performed in *Zostera marina* and *Zostera noltei*, exposing plants from contrasting thermal localities to realistic heat waves (Reusch et al. 2008; Bergmann et al. 2010; Franssen et al. 2011; Winters et al. 2011; Gu et al. 2012; Franssen et al. 2014). In particular, these studies showed that transcriptomic profiles diverged after the heat-stress event (i.e. recovery phase), where e.g. genotypes from the northern sites failed to recover and showed failed metabolic compensation. A differential resilience capacity was also recognized between the two species (see also Chapter II). Similarly, studies on the Mediterranean species *P. oceanica* and *C. nodosa* featured a contrasting thermo-tolerance and capacity to heat acclimation either across species and between depth-related ecotypes (Marìn-Guirao et al. 2016; Marìn-Guirao et al. 2017; Tutar et al. 2017) (see also Chapter II). A recent work by Traboni et al. (2018) identified candidate genes, involved in different phases of the CSR, as possible sub-lethal stress biomarkers of heat stress in *P. oceanica*.

1.3.4 *Multiple-stressors studies in seagrasses*

In seagrasses, a significant knowledge gap exists in multiple-stressor research. In fact, despite the relative number of studies including multiple stressors has increased notably during the last decades (Salo, 2014), most of them are still estimating the impacts of only one stressor per time and the interactive impacts remain understudied. There are few examples where the effect of the combination of multiple stressors is really assessed (Kahn and Durako 2006; Valentine et al. 2006; Koch et al. 2007; Gera et al. 2013; Jiang et al. 2013; Raun and Borum 2013; Villazán et al. 2013; York et al. 2013; Salo and Pedersen 2014; Villazán et al. 2015). More recently, two multiple-stressor studies introduced gene-expression analyses concomitant with physiological and morphological estimations (Ravaglioli et al. 2017; Ceccherelli et al. 2018). Ravaglioli et al. (2017) demonstrated that

nutrient enrichment might mitigate the negative impact of OA on *P. oceanica*, as confirmed by the upregulation of N transporter genes and down-expression of antioxidants. The interactive effects of eutrophication and burial on *P. oceanica* was addressed by Ceccherelli et al. (2018). Notably, they found that the expression of target genes involved in photosynthesis and carbon metabolism had the highest correlation with plant survival and served as anticipatory signals of imminent shoot density collapse.

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Chapter II - Within-shoot variability and leaf-specific stress response in *Posidonia oceanica* exposed to an intense warming event

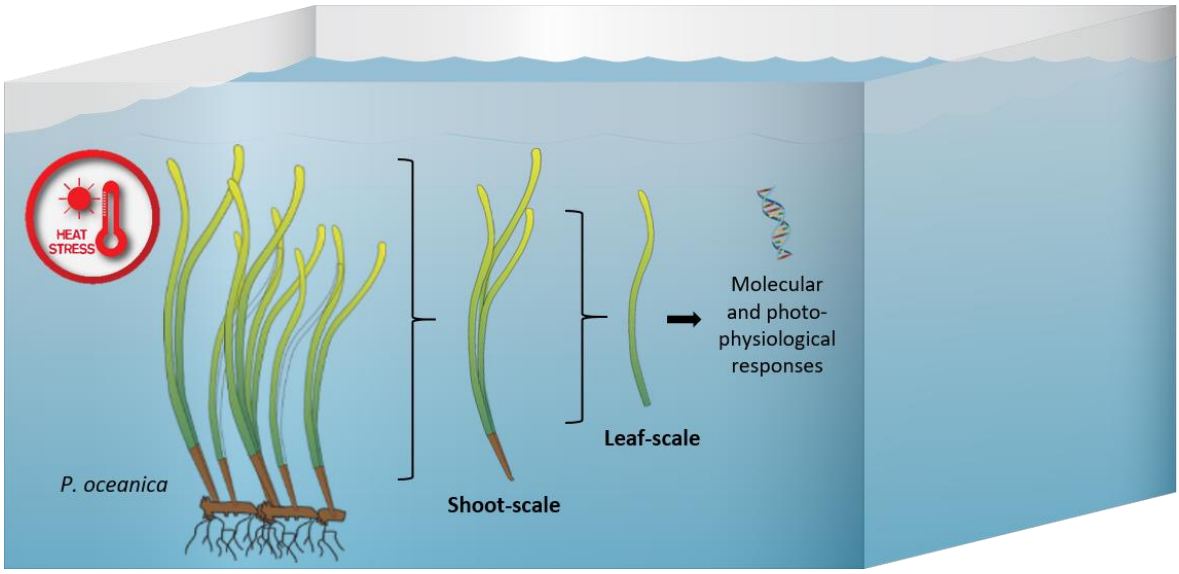

Fig. 2.1 Conceptual diagram of the experiment presented in this chapter. Within-shoot variability and leaf-specific stress response to warming in *P. oceanica*. (All symbols taken from http://ian.umces.edu/imagelibrary/).

The first part of the work presented in this chapter has been published previously:

Miriam Ruocco, Lázaro Marín-Guirao, Gabriele Procaccini (2019) Within- and among-leaf variations in photophysiological functions, gene expression and DNA methylation patterns in the large-sized seagrass *Posidonia oceanica*. Marine Biology 166(3):24

2.1 Introduction

2.1.1 *Variations in physiological and molecular functions along the longitudinal axis of monocot leaves*

In monocot leaves, the establishment of physiological and biochemical functions follows a base-to-tip (basipetal) developmental gradient. Cells divisions occur primarily in the basal meristem, where older cells are progressively displaced by younger ones below them (Martineau and Taylor 1985). As a result, a positional gradient of cell ages along the leaf is formed, with the youngest, undifferentiated and immature cells, at the base of the leaf blade, and the oldest and most mature cells at the tip (Sharman 1942; Evert et al. 1996).

Light-controlled leaf photosynthetic differentiation also proceeds basipetally (Leech et al. 1973; Martineau and Taylor 1985) and involves a dramatic specialization of cell types and plastids, which is based on the activation of a large number of nuclear and plastid genes (Mullet 1988). During its development, the new leaf gains photosynthetic competency, and undergoes a transition from a nutrient sink structure, dependent on imported carbohydrates, to an autotrophic structure (source) that exports photosynthates to other parts of the plant (Evert et al. 1996).

Shifts in physiological and biochemical properties along the longitudinal axis of the leaf are coordinated by well-defined gene expression gradients, as demonstrated by a number of studies that reconstructed high spatial resolution transcriptomes and proteomes of leaf developmental stages in major crop species, such as maize, sugarcane and rice (Cahoon et al. 2008; Li et al. 2010; Majeran et al. 2010; Pick et al. 2011; Chang et al. 2012; Liu et al. 2013; Wang et al. 2014; Li et al. 2015; Mattiello et al. 2015; Yu et al. 2015).

In *Zea mays*, the analysis of transcripts abundance along the developmental gradient of the third leaf, revealed the presence of three major biochemical compartments: the basal part of the leaf, enriched in transcripts encoding enzymes for basic cellular functions such as DNA synthesis, cell wall synthesis, cell cycle regulation and chromatin structure, protein metabolism and hormone signaling; the sink to source leaf transition zone, where there was an increase in the abundance of transcripts associated with the establishment of the photosynthetic machinery, including those required for tetrapyrrole biosynthesis (chlorophyll precursors) and photoreceptors; and the leaf tip, where the transcriptional machinery was almost exclusively dedicated to photosynthesis reactions, including genes for Calvin cycle enzymes, photosystem subunits, and sucrose and starch metabolism-related enzymes and transporters (Li et al. 2010). Similar observations had been made also for the

matured leaf of rise, where a gradual transition from accumulating transcripts associated with primary cell wall formation, basic cellular metabolism and secondary metabolites at the base, to those related to photosynthetic functions and energy production in the middle, and genes involved in catabolic processes toward the tip, was described (Li et al. 2015).

Different leaf developmental stages show also different DNA methylation patterns. DNA methylation is a widespread epigenetic modification in plant genomes mediated by DNA methyltransferase (DMTs), and is known to play a role in developmental programs through the regulation of gene expression (Richards 1997; Zhang et al. 2010). In maize, consecutive developmental zones of the growing leaf were found to exhibit different DNA methylation levels, concomitant to differential expression of maintenance DMTs, and this was associated with expression changes of genes required in the specific developmental context (Candaele et al. 2014).

Seagrasses possess similar organs and tissues as other terrestrial monocots, despite specific adaptations to the marine environment (see Chapter I). Below-grounds parts generally consist of roots for anchoring and rhizomes/stem for mechanical support, while above-ground parts consist of clonal shoots bearing a leaf bundle with a variable number of leaves of different developmental stages. A leaf usually has a basal sheath for protecting the apical meristem and developing leaves, and a distal leaf blade with photosynthetic and transpiration functions (Kuo and Den Hartog 2007). Seagrass leaves grow from the bottom to the top of the canopy, and from inner towards the outer parts of the shoot. As a result, younger parts of a leaf are exposed to lower light intensities than the older apical sections and also inner (i.e. younger) leaves within a plant receive lower light levels than outer (i.e. older) leaves. During its development, each leaf section moves progressively to higher light climates, so it must be shade-acclimated initially, then progressively photo-acclimated to high light conditions (Enríquez et al. 2002; Zimmerman 2007).

In seagrasses, most research has focused on examining shoot and leaf-scale variations in photosynthetic performance. Specifically, several investigations have recognized that photosynthesis and photosynthetic-pigment content are not constant among and within seagrass shoots and vary along a single leaf blade as a function of tissue age, plant size and architecture, and light environment (Mazzella et al. 1980; Alcoverro et al. 1998; Dalla Via et al. 1998; Durako and Kunzelman 2002; Enríquez et al. 2002; Ralph et al. 2002; Olivé et al. 2013). For example, large-sized species as the temperate *Posidonia australis* and the tropical *Thalassia testudinum* have clear photochemical patterns along their leaves (Ralph and Gademann 1999; Enríquez et al. 2002), but with some differences derived from the different light environments in which they grow. The medium-sized *Z. marina*, instead,

shows no photosynthetic nor pigmentary changes along the leaf blade (Ralph et al. 2002). Despite these studies, the understanding of how physiological and biochemical functions vary among and within seagrass leaves is much limited when compared to terrestrial monocots, while molecular processes underlying such functions have never been investigated so far.

2.1.2 *Heat stress response in terrestrial and marine plants*

Warming of the climate because of the greenhouse effect has become a major concern. Following the increase in mean value of temperatures, episodes of climate extremes (e.g. heat waves) are expected to occur more frequently and with higher intensity and duration (Christoph and Gerd 2004; Lewis and King 2017). From an ecological perspective, such changes in temperature variance will have a disproportionately greater effect on species' performance than changes in the mean (Vasseur et al. 2014). Acting as heat sinks, global oceans absorb most of this extra energy from the atmosphere. As a results, the average temperatures of the upper layers of the oceans have increased of about 0.7 °C over the last 100 years, with direct effects on marine ecosystems and biogeochemical processes (Hoegh-Guldberg and Bruno 2010; Gruber 2011), and a further increase of 1-3.5 °C is expected by the end of the century (IPCC 2007; IPCC 2014).

Much research on the heat stress response (HSR) has been conducted in terrestrial higher plants, particularly in economically and dietary important crops, since extreme high temperatures are major threats for agricultural production and food safety (Röth et al. 2015). Heat stress adversely affects plant growth, development, physiological processes, and yield (see Hasanuzzaman et al. 2013 for a review). At molecular level, the processes of sensing and responding to heat stress comprise the activation of numerous regulatory and signaling pathways that eventually lead to metabolic adjustments to minimize the damage and re-establish the cellular homeostasis (Kotak et al. 2007). Currently, the production of massive datasets from *omics* studies have greatly advanced the understanding of HSR, leading to the identification of new heat stress-responsive genes and proteins, or even whole new pathways (Qu et al. 2013; Ohama et al. 2017). In general, HS responsive genes/proteins can be divided into two distinct groups: signaling components that regulate gene expression responses, such as protein kinases and transcription factors (TFs); and functional components, that directly protects plant cells against heat stress, including heat shock proteins (HSPs) and antioxidant enzymes (e.g. APX and CAT) that act as ROS scavengers (Qu et al. 2013). In addition to expressing general stress-responsive genes/proteins, extensive reprogramming of primary

and secondary plant metabolism occurs in response to heat stress (Allakhverdiev et al. 2008; Wang et al. 2018).

In contrast to terrestrial plants, knowledge about the effects of warming on marine plants is limited, as for the tolerance mechanisms they can activate to overcome short exposures to temperature extremes. Although seagrasses possess similar gene repertoires to respond to heat stress as their terrestrial counterpart (Franssen et al. 2011; Marín-Guirao et al. 2017), they may react differently due to the peculiarity of thermal stress in the aqueous medium (Feder and Hofmann 1999).

In the last years, a series of common garden experiments have been conducted in *Z. marina* and *Z. noltei*, exposing individuals from contrasting thermal localities (i.e. northern and southern European populations) to simulated heat stress (Bergmann et al. 2010; Franssen et al. 2011; Winters et al. 2011; Gu et al. 2012; Franssen et al. 2014; Jueterbock et al. 2016). These studies emphasized a greater similarity in gene expression and physiological responses during the exposure period, followed by a contrasting response after the heat wave, with high-latitude populations failing to recover from the stress (Franssen et al. 2011; Winters et al. 2011). A differential resilience capacity was also identified between *Z. marina* and *Z. noltei*. Specifically, when exposed to the same heat wave scenario, *Z. marina*, often dominant in subtidal environments and subjected to lower temperature extremes, and *Z. noltei*, predominantly intertidal, showed markedly different transcriptomic responses, reflecting the higher thermal tolerance of *Z. noltei* (Franssen et al. 2014).

The Mediterranean basin is considered one of the most sensitive oceanic regions regarding to global warming and climate extremes, and it is predicted to warm at rates twofold faster than global oceans (IPCC 2007; Jordà et al. 2012). Increases of 4-5 °C in maximum summer temperatures have been predicted in some areas, accompanied by an increase in the frequency and intensity of heat waves (Sánchez et al. 2004; Jordà et al. 2012; Olsen et al. 2012). In addition, extremely high temperatures can be reached in confined waters like coastal lagoons, where water temperatures are often naturally beyond the theoretical tolerance limits of the species (Tomasello et al. 2009).

First studies on the key Mediterranean species *P. oceanica* and *C. nodosa*, featured intra and inter-specific variability in molecular and physiological responses to short-term heat stress (Marín-Guirao et al. 2016; Marín-Guirao et al. 2017; Tutar et al. 2017). In *P. oceanica*, a contrasting tolerance and capacity to heat acclimation was found for individuals collected along a depth gradient. Shallow genotypes were able to acclimate to warming through respiratory homeostasis and activation of photo-protective mechanisms, whereas deep genotypes experienced photosynthetic injury and impaired carbon balance (Marín-Guirao et

al. 2016). At transcriptional level, this was supported by a stronger activation of heat-responsive genes (e.g. HSPs and antioxidants), as well as genes involved in photosynthesis and respiration, in shallow genotypes, while deep ones activated amino acid/sugar metabolic processes, and ubiquitination/proteolysis-related genes, suggesting extra-energy needs and severe protein damages (Marín-Guirao et al. 2017). Interestingly, epigenetic regulation of gene expression through DNA methylation and chromatin remodeling, seem to have a key role in seagrass adaptive responses to heat stress, as evidenced by the induction of genes involved in DNA and histone methylation (Marín-Guirao et al. 2017). Diverging mechanisms of heat acclimation were also found between *P. oceanica* and *C. nodosa*, with the former species achieving a complete metabolic homeostasis through the regulation of photosynthesis and respiration processes at the level of control plants, while the latter balancing both processes at enhanced rates (Marín-Guirao et al. 2016).

More recently, the carbon economy of *Posidonia* and *Cymodocea* from contrasting thermal environments was addressed in a six-week simulated heat wave experiment (Marìn-Guirao et al. 2018). This study revealed that the strategies through which these plants acclimate to warming is also determined by their ability to modify the proportion of fixed carbon that is destined to main plant carbon sinks: respiration, growth and storage. These strategies differed between species due to their inherent biological attributes (large size and slow-growing *vs*. medium size and fast-growing) and to the thermal environment where they grow (cold *vs*. warm) (Marìn-Guirao et al. 2018).

Finally, a novel study investigated the different phases of the cellular stress response (CSR) in *P. oceanica* exposed to increasing temperature (20°C to 32°C). A molecular traffic light was proposed as a response model including green (protein folding and membrane protection), yellow (ubiquitination and proteolysis) and red (DNA repair and apoptosis) categories (Traboni et al. 2018). Gene-expression analysis revealed that molecular chaperoning, DNA repair and apoptosis inhibition processes-related genes were the ones that mostly responded to high-thermal stress (Traboni et al. 2018).

All these studies have been conducted generally considering the averaged response of the whole plant or mature leaf tissues (generally the middle section of leaf rank 2 or 3 of the shoot). Fine-resolution studies aiming at understanding the differential response to heat stress of different plant/leaf developmental stages, or among different organs, are currently completely missing in seagrasses.

2.1.3 *The study*

The understanding of how physiological and biochemical functions vary within and among seagrass leaves due to developmental/environmental cues, and in response to stress, is much limited when compared to terrestrial monocots, as for the underlying molecular mechanisms. The study presented in this chapter has a dual aim: I) disentangle how gene expression patterns modulate the functional specialization of specific leaves/leaf segments, representing different developmental stages, and responsible for variable photosynthetic capacity; II) investigate how molecular and photo-physiological responses to a heat stress vary within the same tissue, as a function of the specific developmental/environmental context.

To address these questions, the seagrass *P. oceanica* was exposed to a short-term acute heat stress for one week in a mesocosms system. Control samples were maintained to light/temperature levels resembling environmental conditions experiences by the natural population during the study period. The expression gradients of selected genes associated to key plant metabolic processes (photosynthesis, chlorophyll biosynthesis, mitochondrial respiration, general stress response and programmed cell death), were determined in three sections (i.e. basal, medium, high) established along the longitudinal axis of three leaves of different age (i.e. youngest, young and mature) within the shoot, in both control and heated treatments. Shifts in target gene expression were correlated with chlorophyll *a* fluorescence-derived photosynthetic parameters and pigment content (Chl*a*, Chl*b*, total carotenoids) of the same leaf segments. Plant morphological attributes and fitness-related traits (leaf growth rate and necrotic surface) were also determined. Finally, I estimated the global DNA methylation (5-mC) level in different leaf developmental stages and under heat stress.

2.2 Materials and Methods

Experimental design

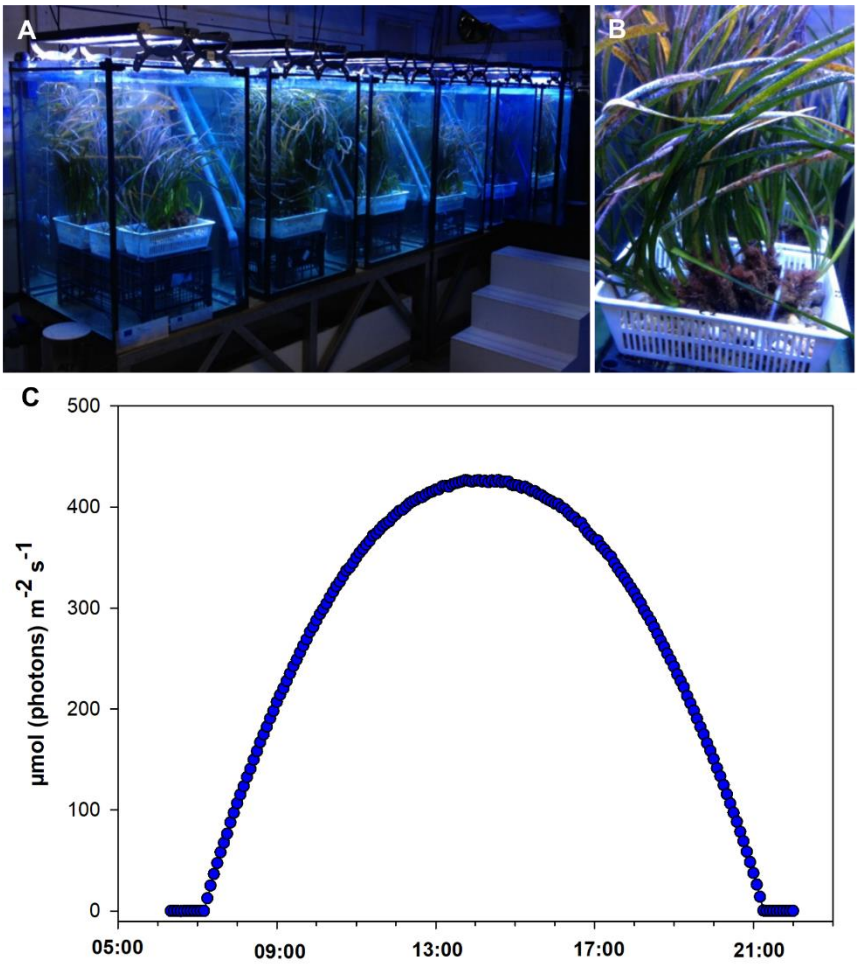

Fig. 2.2 (A) View of the mesocosm system at SZN; (B) example of a *P. oceanica* ramet attached to the plastic cage; (C) daily cycle of PAR irradiance measured in the experimental tanks. Photo credit: M. Ruocco.

For this study, large *P. oceanica* fragments (i.e. ramets), consisting of a horizontal rhizome bearing numerous vertical shoots, were collected by SCUBA diving from a shallow-water meadow (8-10 m depth) located around the island of Procida (Gulf of Naples, Italy 40°45.218'N, 14° 01.400' E) on 5th July 2016 (11:00-12:00 pm). Seawater temperature in the study area annually ranges between 13.82 °C and 28.96 °C (data from 2013-2015) and 14.37 °C and 28.55 °C (data 2016-2018), with an average T of 18.12 °C and 18.75 °C, respectively. The temperature of 28.96 °C was the max recorded in July 2017 (data from ARPAC DT – U.O MARE; http://www.arpacampania.it) for the Gulf of Naples (station codes 15-NA006 and 15 NA005). The distance between sampled plants within the meadow was > 5 m to ensure sufficient genetic diversity. Plant material was kept in darkened coolers

filled with ambient seawater and rapidly transported to the laboratory (within 1-2 hr) to be immediately transplanted in an indoor mesocosm facility at Stazione Zoologica Anton Dohrn (Naples, Italy) (Fig. 2.2 A). Twelve plant fragments of similar size and shoot number (15-20 connected shoots) were selected to standardise the experiment, and individually attached to the bottom of twelve plastic net cages (34x24x10 cm) filled with coarse sediment (Fig 2.2 B). Two randomly selected cages were placed in each of six independent aquaria (500 L) (Fig. 2.2 AB). Large rhizome fragments of *P. oceanica* were preferred over small ones to ensure the optimal conditions of plants during the experimental period (Marín-Guirao et al. 2013) and to maintain the canopy structure of the meadow, responsible for regulating the light gradient from the top to the base of plants (Sandoval-Gil et al. 2014). The position of the two pots inside the aquaria was periodically changed to avoid the influence of spatial micro-gradients of unknown factors. Each aquarium was equipped with its own illumination system designed *ad hoc* for the laboratory facility, and consisting of two LED lamps allowing the simulation of light spectra from 0 to 30 m depth, light circadian fluctuation, and light intensities up to 800 µmol photons m^{-2} s^{-1} below the water surface (M2M Engineering, Naples, Italy). The light source created a highly homogenous field of irradiance across each tank. Water temperature in aquaria was controlled by aquarium chiller/heaters (Teco TK 2000). Seawater quality was maintained through continuous mechanical filtration and UV sterilization. Aquaria and filters were cleaned every day in order to prevent the appearance of epiphytes and macroalgal blooms. Continuous light and temperature measurements were performed using a LI-COR LI-1400 quantum sensor (Fig. 2.2 C) and HOBO® Pendant® UA-002-64 data loggers (Onset Computer Corporation), respectively. Salinity was measured daily using a WTW Cond 3310 portable conductivity meter and kept along the experiment within the range of 37.3–37.7 psu by adding freshwater to compensate for evaporation.

In order to remove any stress associated to sampling and transplantation, plants were maintained during one week (i.e. acclimated) under the same mean environmental conditions experienced by the natural population during the study period (temperature: ca. 25 °C; max. noon irradiance: ca. 400 µmol photons m^{-2} s^{-1} above the canopy; 12 h:12 h light:dark photoperiod). Subsequently, temperature in half of the tanks was increased to 34 °C to induce an acute heat stress (Fig. A2.1 in Appendix II). Plant exposure lasted one week. One randomly selected vertical shoot (avoiding apical ones) from each of the twelve pots was sampled to analyze variations in photo-physiology, pigment content and gene expression, among and within *P. oceanica* leaves. From each shoot, the leaves 1 (youngest), 2 (young) and 3 (mature) were detached and three sections along their longitudinal axis were established: B (Basal) – the lowest portion of the leaf at 5 cm distance from the ligule; M

(Medium) – the intermediate section of the leaf at 20 cm distance from the ligule; H (High) – the upper section at 40 cm distance from the ligule. A 3-cm tissue section above and below the established heigth was collected. The newborn leaf of the shoot (< 3 cm) was discarded due to its small size, as were rank leaves 4 and 5, due to the high epiphytic cover, the presence of nectrotic marks and broken tips. Photo-physiological and molecular responses were determined on two shoots per tank (one per pot). Within each tank, all measurements were averaged, since the tank is considered as the true experimental replicate. This means that the number of replicates used in statistical tests was *n*=3 (total biological replicates N=6).

Shoot morphology and growth

At least four vertical shoots per tank were randomly chosen to characterize plant morphology (i.e. number of leaves, leaf length, and necrotic surface). Leaf growth rate was determined using the Zieman method (Zieman 1974), that is by marking the boundary limit between the leaf and the ligule with a fine needle. Shoots were marked right after the acclimation phase at the onset of the experimental treatment, then they were subsequently collected at the end of the thermal exposure to estimate the surface area of newly formed tissue (below the mark) and thus to infer the leaf growth rate. Within each tank, measurements were averaged to be used as independent replicates (*n*=3).

Photo-physiology and pigment content

Chlorophyll *a* fluorescence measurements were performed with a diving-PAM portable fluorometer (Walz, Germany) as described in Marín-Guirao et al. (2013). The saturation pulse method was used to measure the basal (F_0) and maximum fluorescence (Fm) and to calculate the maximum photochemical efficiency of PSII [$(Fv/Fm = (Fm-F_0)/Fm$] in the selected leaf segments of plants adapted to the dark throughout the night. The rapid light curve (RLC) method was subsequently applied on the same leaf segments at noon, after 5 hours under illumination in the aquaria and each curve involved a 20-s exposure to 9 incremental irradiances. The relative electron transport rate (r-ETR) was obtained from the PAM WinControl program (Walz, Germany), and non-photochemical quenching was calculated as NPQ = (Fm–Fm')/Fm' where Fm' is the maximum fluorescence of light-adapted leaves.

Following the chlorophyll *a* fluorescence measurements, the analyzed leaf segments (108 in total) were then stored in complete darkness at -80 °C for pigment analysis. Pigment extraction was then carried out by homogenizing 1-cm^2 leaf segments in 80% acetone,

buffered with MgCO3 solution to prevent acidification of the extract (Dennison, 1990). Extracts were stored at 4 °C in the dark for 24 h and subsequently centrifuged (1000g for 10 m at 4°C). The absorbance of the extracts was then determined spectrophotometrically at 470 nm, 646 nm, 663 nm and 725 nm, using a 1 mL quartz-glass cuvettes. The chlorophyll *a* and *b* concentrations, as well as the total carotenoid concentration, were calculated using the equations defined by Lichtenthaler and Wellburn (1983), and expressed as μg cm^{-2}.

Gene expression

Leaf sub-samples for gene expression analysis were collected from the same plants and leaf sections exploited for the pigment analysis (108 samples). Plant material was gently cleaned from epiphytes and entirely submerged in RNAlater© tissue collection (Ambion, life technologies), stored one night at 4°C to let the solution penetrate into the tissue, and finally stowed at -20°C until RNA extraction. Total RNA was extracted with Aurum™ Total RNA Mini Kit (BIO-RAD) following manufacturer's instructions. The purity and concentration of RNA was checked using NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific) and quality was assessed using 1.0% (w/v) agarose 0.5X TBE gel (0.5 mg/mL EtBr) electrophoresis. Five hundred nanograms of RNA from each sample were retro-transcribed in cDNA with the iScript™ cDNA synthesis kit (BIO-RAD), according to manufacturer's protocol.

Primers for putative Reference Genes (RGs) and Genes of Interest (GOIs) have been designed from *P. oceanica* transcriptomes (D'Esposito et al. 2017; Marín-Guirao et al. 2017) with the primer analysis software Primer3 v. 0.4.0 (Koressaar and Remm 2007; Untergasser et al. 2012), or selected from previous studies (see Table 2.1). Design conditions included primer length (18-23 bp), T_m (~60°C), GC content (≥ 50%) and product size (100 to 200 bp). A number of genes involved in light reaction function of photosynthesis (*psbA*, *psbD*, *psbC*, *PSBS* and *FD)* and carbon fixation (*RBCS*), chlorophyll *a-b* binding proteins (*CAB-151*), and genes related to chlorophyll biosynthesis (*POR*) and mitochondrial energy dissipation mechanisms (*AOX*), were targeted. General stress-responsive genes, such as heat shock proteins (*HSP90* and *SHSP*) and regulator of programmed cell death (*BI*) were also selected. Three putative RGs (*18S*, *eIF4a* and *L23*) were chosen and tested for stability in the experimental conditions, on the basis of previous works conducted in the same species under several abiotic stresses (Serra et al. 2012; Dattolo et al. 2014; Lauritano et al. 2015; Marín-Guirao et al. 2016). For full gene names, see Table 2.1.

Reverse Transcription-quantitative PCR (RT-qPCR) reactions were performed in MicroAmp Optical 384-well reaction plates (Applied Biosystems) using Fast SYBR® Green

Master Mix (Applied Biosystems) as detection chemistry and Viia7 Real Time PCR System (Applied Biosystems). Reactions were carried out in a 10µl final volume with 5µl MM, 2µl of 1.4 pmol μl^{-1} primers and 3µl of 1:30 cDNA template and assembled in the 384-well plates format, by means of a Tecan Freedom EVO® 200 automated liquid handling system. Thermal profile was the following: 95°C for 20 s, 40 times 95°C for 1s and 60°C for 20s. For determining the specificity of the reaction, the melting curve of each amplicon from 60 to 95°C was also detected. All RT-qPCR reactions were conducted in triplicate and each assay included also three no-template negative controls. The technical variation among the triplicates was checked and individual outliers were excluded when SD was higher than 0.3. RT-qPCR efficiencies for all primer pairs were calculated from the slopes of standard curves of the threshold cycle (C_T) *vs.* cDNA concentration (at least five dilution points), with the equation $E = 10^{-1/slope}$. Primer's sequences, percent efficiencies (*E*) and regression coefficients (R^2) of RGs and GOIs are reported in Table 2.1. Three different algorithms were utilized to identify the best RGs in our experimental conditions: BestKeeper (Pfaffl et al. 2004), geNorm (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004).

Table 2.1 List of Reference Genes (RGs) and Genes of Interest (GOIs) assessed in *P. oceanica* using RT-qPCR. Gene and protein names, primer sequences, amplicon size (S, base pair), percent efficiency (*E*), correlation coefficient (R^2) and references, are given.

Gene	Protein	Primer Sequences 5'→3'	S	*E*	R^2	Reference
Reference genes						
18S	Ribosomal RNA 18S	F:AACGAGACCTCAGCCTGCTA R:AAGATTACCCAAGCCTGTCG	200	100%	0.99	Serra et al. 2012
eIF4A	Eukaryotic initiation factor 4A	F: TTCTGCAAGGGTCTTGACGT R:TCACACCCAAGTAGTCACCAAG	192	100%	0.99	Lauritano et al. 2015
L23	60s ribosomal protein L23	F:AAAGATACAGGCTGCCAAGG R:TGGTCCAACTTGTTCCTTCC	168	100%	0.99	Serra et al. 2012
Genes of interest						
psbA	Photosystem II protein D1	F:GACTGCAATTTTAGAGAGACGC R:CAGAAGTTGCAGTCAATAAGGTAG	136	92%	0.99	Dattolo et al. 2014
psbD	Photosystem II protein D2	F:CCGCTTTTGGTCACAAATCT R:CGGATTTCCTGAGAAACGAA	161	100%	0.98	Dattolo et al. 2014
psbC	Photosystem II CP43 reaction center protein	F: TTTCATCGCTTGTTGTTTCG R:ATGTTAGCCCCAAGACGTTG	135	93%	0.99	This study
PSBS	Photosystem II 22 kDa protein	F:CCGCTCCTGTTGTTCTTCAT R:GGACCTCCTTCCTTGAGACC	158	100%	0.99	Dattolo et al. 2014
FD	Ferredoxin-1, chloroplastic	F:TCAGACTGGGGGTAAGCAAC R:TCTACATCCTCGACCACTGC	187	100%	0.98	Dattolo et al. 2014
RBCS	RuBisCO small subunit	F:AGCATGGTAGCACCCTTCAC R:GGGGGAGGTATGAGAAGGTC	169	100%	0.99	Dattolo et al. 2014
CAB-151	Chlorophyll *a-b* binding protein 151, chloroplastic	F:AAGCCCATTAGCACAACCTG R:GGGCAATGCTTGGTACTCTC	199	93%	0.99	Dattolo et al. 2014
POR	Protochlorophyllide reductase	F: AGTTCCACAGACGGTTCCAC R:AATCACCACCTGAGCGAGTC	194	98%	0.99	Ruocco et al. 2018

AOX	Alternative oxidase 1a	F: TGCTGCATTGCAAGTCTCTAC R: GTTGTGACACCTCCATGAAGGTC	116	100%	0.99	Procaccini et al. 2017
BI	Bax inhibitor 1	F: CCCGTGGAACTACTTGCTGT R:GGAATGCAGCCTCCAGAATA	107	100%	0.98	Traboni et al. 2018
HSP90	Heat shock protein 90	F: CTCCATCTTGCTTCCCTCAG R:TCAGTTTGGAGGAACCGAA	146	100%	0.99	Lauritano et al. 2015
SHSP	Small heat shock protein	F: ACCGGAGGATGTGAAGATTG R:AGCTTGCTGGACAAGGTGAT	125	99%	0.98	Lauritano et al. 2015

DNA methylation

Leaf material for DNA extraction was obtained from the following leaf sections: leaf 1 – Basal, leaf 2 – Medium, and leaf 3 – High, of six different ramets (one per tank) in control and heated conditions (*n*=3). Leaf tissue (about 5 cm) was accurately cleaned of epiphytes and dried with silica gel. Genomic DNA was isolated using the NucleoSpin® Plant II kit (Macherey-Nagel) following manufacturer's instructions. DNA quality was assessed through 1.0% (w/v) agarose 0.5X TBE gel (0.5 mg/mL EtBr), DNA purity was estimated using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific) and concentration was accurately determined by the Qubit dsDNABR assay kit using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific). Global DNA methylation was assessed colorimetrically in duplicate by an ELISA-like reaction with the MethylFlash™ Methylated DNA Quantification Kit (Epigentek Inc.), and reported as % methylated DNA (5-mC) relative to the input DNA quantity for each leaf section. Fifty nanograms of DNA per sample were analyzed. Absorbance at 450 nm was assayed using a Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific).

Data analysis

Relative quantification of gene expression was obtained following Livak and Schmittgen (2001). In details, after normalizing by the efficiency established for each primer pair (see Table 1), the negative differences in cycles to cross the threshold value between the RGs and the respective GOI (-ΔCT) were calculated according to equation (1). Mean -ΔCT values were then calculated for biological replicates of each leaf rank (i.e. 1, 2 and 3), leaf height (i.e. Basal, Medium and High) and treatment (Control and Heated), from individual -ΔCT values. Data collected from the two *P. oceanica* fragments placed in each tank were averaged (*n*=3). Fold expression changes were definitely obtained with the equation (2):

(1) $-\Delta CT = CT_{RGs} - CT_{GOI}$

(2) Fold expression change = $\pm 2^{(|(-\Delta CT\ \text{treatment}) - (-\Delta CT\ \text{control})|)}$

Two different analyses were conducted: the "Control" analysis, using only data obtained from control samples, to assess the natural variability in photo-physiological and molecular functions among and within *P. oceanica* leaves; and the "Control *vs.* Heated" analysis, using the full dataset, to assess the fine-scale heat stress response of *P. oceanica*. Multivariate statistics was used to assess the overall signal of all photo-physiological variables

(photosynthetic parameters and pigment content) and GOIs. Specifically, a Permutational Multivariate Analysis of Variance (PERMANOVA) was conducted with the Primer 6 v.6.1.12 & PERMANOVA + v.1.0.2 software package (PRIMER-E Ltd) (Clarke and Gorley 2006). The "Control" analysis consisted of two fixed factors: Leaf Rank (LR), with three levels (1, 2 and 3) and Leaf Height (LH), with three levels (Basal, Medium and High); the "Control *vs.* Heated" analysis consisted of three fixed factors: Leaf Rank (LR) and Leaf Height (LH) (with the same aforementioned levels), and Treatment (T), with two levels (Control and Heated). Principal Component Analyses (PCA) were also performed for the multivariate photo-physiological and gene expression datasets with the software PAST v.3.03 (Hammer et al. 2001). Following, a two-way and three-way Analyses of Variance (ANOVA) were conducted for the "Control" and "Control *vs.* Heated" analyses, respectively, to detect the effects of leaf rank, leaf height and treatment on single photo-physiological variables and individual gene expression. Global DNA methylation data were analyzed by one and two-way ANOVA for the "Control" and "Control *vs.* Heated" analyses, respectively. Differences in relative leaf growth rate and necrosis between control and heated plants were tested by a Student's *t*-test. Normality of data was checked using the Shapiro-Wilk test and variance homogeneity was verified using Levene's test. When parametric assumptions were not met, data were Box-Cox transformed. Student-Newman-Keuls post-hoc test was used whenever significant differences were detected. All ANOVAs were performed using the statistical package STATISTICA (StatSoft, Inc. v. 10). Relationships among the different molecular and photo-physiological parameters were also explored through Pearson's correlation analyses. To graphically visualize correlations between gene expression and photo-physiological patterns, heatmaps were generated in R using the heatmap.2 function from the gplots package (http://CRAN.R-project.org/package=gplots).

2.3 Results

Best reference gene (RG) assessment

Three putative RGs (*18S*, *eIF4A* and *L23*) were chosen and tested for stability in different leaf sections of *P. oceanica,* and under heat stress (Table 2.1). The two algorithms Beestkeeper and geNorm agreed in suggesting *L23* and *18S* as the best reference genes in our experimental conditions (see Tables 2.2 and 2.3), while NormFinder approach indicated *18S* as the most stable gene, with the same stability value of *eIF4A* (see Table 2.4). However, since in the geNorm analysis *eIF4A* showed an average expression stability (*M*) much higher than the threshold of 1.5, which indicates a suitable RG, this gene was discarded from the RG panel. Therefore, only *18S* and *L23* were used for the normalization of the target gene expression dataset.

Table 2.2 Selection of reference genes in *P. oceanica* based on Bestkeeper. Best candidate genes, with lowest standard deviation (SD) of CT values, are underlined.

Gene name	SD [± CT]
L23	0.58
18S	0.58
eIF4A	0.64

Table 2.3 Expression stability of candidate RGs as calculated by geNorm in *P. oceanica*. Best candidate genes, with the lowest average expression stability, are underlined.

Gene name	Average expression stability (*M*)
L23/18S	0.89
eIF4A	3.17

Table 2.4 Expression stability of candidate RGs as calculated by NormFinder in *P. oceanica*. Best candidate genes, with the lowest stability value, are underlined.

Gene name	Stability value	Standard error
18S	0.16	0.02
eIF4A	0.16	0.02
L23	0.17	0.02

2.3.1 Natural photo-physiological and gene-expression variations within and among P. oceanica leaves

Plant morphology

Plants contained a mean of 5 ±0.17 leaves per shoot. Newborn leaves (i.e. 1 in Fig. 2.3A) had a mean length of 2.65 ±0.54 cm and were not utilized in this study. The youngest, young and mature leaves of the shoots (i.e. 2, 3 and 4 in Fig. 2.3A) had a mean length of 45.14 ±6.57, 67.09 ±4.07 and 67.50 ±3.84, respectively, and were those selected for molecular and photo-physiological assessments. Older leaves (i.e. 5 and 6 in Fig. 2.3A), with a mean length of 59.53 ±3.76 and 49.88 ±2.38, presented high epiphytic cover, a 16-18 % necrotic surface and broken tips (100%), thus they were discarded for the analyses. All along the text I refer to rank leaves 2, 3 and 4 (Fig. 2.3A) as leaves 1, 2, and 3, respectively (Fig. 2.3B).

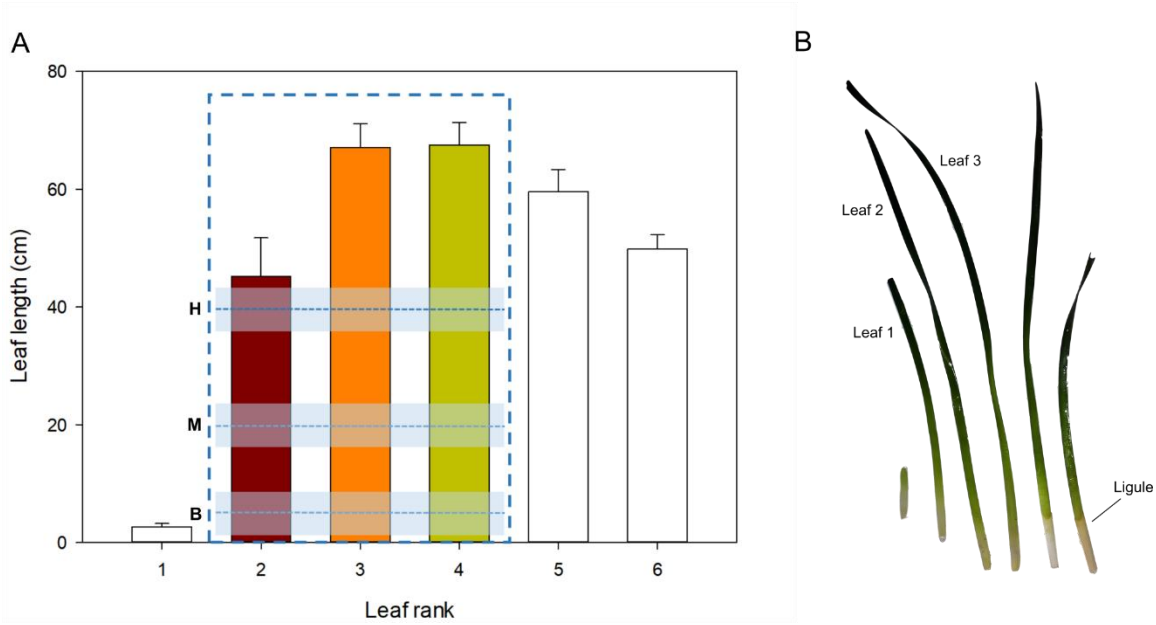

Fig. 2.3 In (A) length of *P. oceanica* leaves (data are mean ± SE). Colored bars represent leaves selected for the experiment. Leaf sections used for molecular and photo-physiological analyses were taken at 5 (B), 20 (M) and 40 (H) cm from the ligule. In (B) an example of a *P. oceanica* shoot collected for the experiment. Targeted leaves are indicated as Leaf 1, 2 and 3.

Photosynthetic parameters and pigment content

Leaf height significantly affected the photo-physiological response of *P. oceanica*, as indicated by the PERMANOVA ($P_{(perm)}$ <0.001; Table 2.5). Pair-wise comparisons emphasized significant differences among all selected leaf sections, with major changes between basal and upper segments (B *vs.* M and H, $P_{(MC)}$ <0.001) and minor changes between middle and high sections (M *vs.* H, $P_{(MC)}$ <0.05).

The PCA clearly separated leaf height groups along the component 1, explaining most of the total variance (68.01 %) (Fig. 2. 4A). Basal segments of selected leaves clustered on the left side of the plot, whereas all medium segments grouped in the middle, and tip sections on the right side. Among photo-physiological variables, the relative electron transport rate (r-ETR), the non-photochemical quenching (NPQ), and pigment content (Chl*a*, Chl*b*, and carotenoids) were positively correlated with the axis 1. Leaf rank groups distributed mostly along the axis 2 of the PCA (19.06 % total variance) (Fig. 2.4A). The basal fluorescence (F_0) was the variable contributing most to this separation (Table 2.6).

Two-way ANOVA confirmed that all analyzed chlorophyll *a* fluorescence-derived photosynthetic parameters varied according to leaf height and/or rank. Basal fluorescence (F_0) was significantly affected by LR (P <0.001; Table 2.7), as it was largely higher in the mature leaf compared to younger ones (1=2≠3; Fig. 2.5). On the contrary, maximum quantum yield (Fv/Fm) was higher in younger than mature leaves (1=2≠3; Fig. 2.5), and varied according to leaf height, decreasing from the base to the upper segments (B=M≠H; Fig. 2.5). The electron transport rate (r-ETR) exhibited the opposite behavior, increasing from the base to medium and tip sections (B≠M=H; Fig. 2.5) without significant changes among the different leaves of the shoot. The non-photochemical quenching (NPQ) was significantly affected by leaf height and rank (P <0.001 for LH and P <0.05 for LR; Table 2.7). It gradually increased from the basal to the upper leaf portions (B≠M≠H) and from younger to mature leaves, with lower values in the second leaf of the shoot (Fig. 2.5).

Photosynthetic pigment content of *P. oceanica* (Chl*a*, Chl*b* and carotenoids) varied according to leaf height (Table 2.7 and Fig. 2.6). Basal leaf sections contained significantly less chlorophylls and carotenoids than middle and upper segments (B≠M≠H; Fig. 2.6). The antenna size (Chl *b*/*a*) did not change depending on leaf height or rank (Fig. 2.6).

Table 2.5 Results of 2-way PERMANOVAs conducted on multivariate gene expression data (-ΔCT values) and photo-physiological variables (photosynthetic parameters and pigment content). $P_{(perm)}$ <0.05 are in bold.

Two-way PERMANOVA					
Photo-physiology					
Source	**df**	**Pseudo-F**	$P_{(perm)}$	**Unique perms**	*Pair-wise tests*
Leaf Height	2	23.742	**0.0001**	9944	LH: B ≠ M ≠ H
Leaf Rank	2	0.1687	0.9677	9952	
LH×LR	4	0.43729	0.8993	9943	
GOIs					
Source	**df**	**Pseudo-F**	$P_{(perm)}$	**Unique perms**	*Pair-wise tests*
Leaf Height	2	11.202	**0.0001**	9933	LH: B ≠ M ≠ H
Leaf Rank	2	5.0391	**0.0006**	9929	LR: 1 ≠ 2 = 3
LH×LR	4	1.518	0.1158	9933	

Table 2.6 Loadings of photo-physiological variables and GOIs on PC1 and PC2 of PCAs depicted in Fig. 2.4 AB. Loadings of variables contributing most to the principal components are in bold.

Photo-physiology	**PC1**	**PC2**	*GOIs*	**PC1**	**PC2**
F_v/F_m	**-0.34**	**-0.42**	**psbC**	0.21	0.22
F_0	0.21	**0.64**	**HSP90**	-0.05	-0.06
r-ETR	**0.40**	-0.22	**SHSP**	-0.03	-0.01
NPQ	**0.39**	**0.25**	**psbD**	0.28	0.10
Chl *a*	**0.41**	-0.19	**CAB-151**	-0.07	**0.47**
Chl *b*	**0.41**	-0.13	**AOX**	**0.53**	-0.30
Carotenoids	**0.41**	-0.19	**BI**	**0.46**	-0.16
Chl *b/a*	-0.17	**0.47**	**FD**	0.27	**0.39**
			POR	-0.14	**0.50**
			psbA	0.19	0.17
			PSBS	**0.48**	0.15
			RBCS	0.12	**0.40**

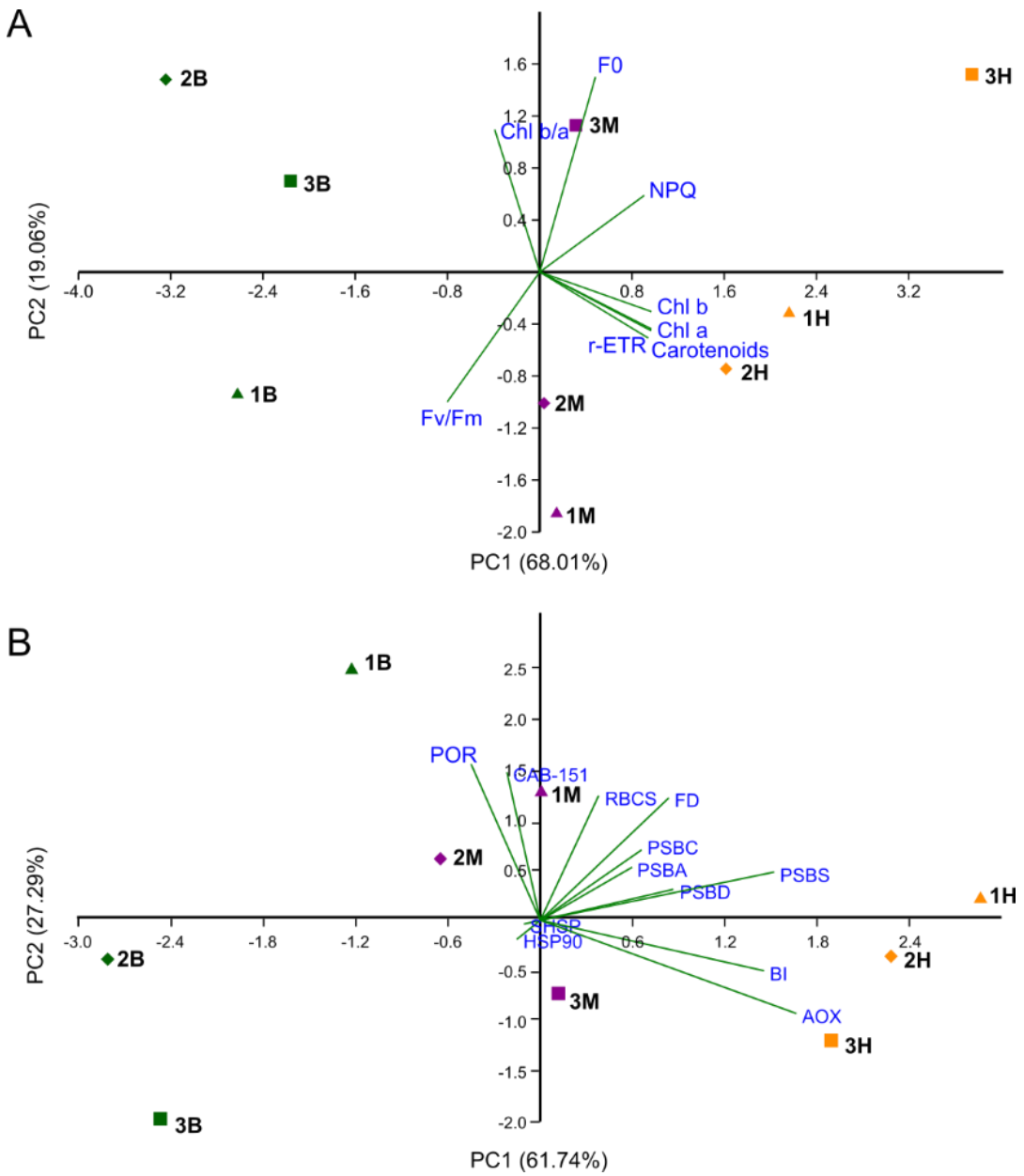

Fig. 2.4 PCA conducted on (A) all photo-physiological variables (photosynthetic parameters and pigment content); and (B) averaged -ΔCT values of individual GOIs. Different colors refer to leaf heights (green = Basal, violet = Medium and orange = High). Different symbols refer to leaf ranks (filled triangles = 1, filled diamonds = 2, filled squares = 3).

Table 2.7 Results of two-way ANOVAs to assess the individual contribution of photo-physiological variables (photosynthetic parameters and pigment content) and GOIs. *P* <0.05 are in bold, *P* <0.1 are underlined. For SNK pairwise results see graphs in Figs. 2.5-8.

Two-way ANOVA

Photo-physiology		**F_0**		**F_v/F_m**		**r-ETR**		**NPQ**		**Chl *a***		**Chl *b***	
Effect	**df**	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***
Leaf Height	2	2.825	0.086	10.705	**0.001**	18.044	**0.000**	25.647	**0.000**	26.413	**0.000**	23.916	**0.000**
Leaf Rank	2	15.375	**0.000**	8.034	**0.003**	0.028	0.973	3.633	**0.047**	0.151	0.861	0.038	0.963
LH×LR	4	1.392	0.277	0.471	0.756	1.156	0.363	0.652	0.633	0.120	0.974	0.079	0.988

		Carotenoids		**Chl *b*/*a***	
		F	***P***	**F**	***P***
Leaf Height	2	37.026	**0.000**	0.579	0.570
Leaf Rank	2	0.126	0.883	2.510	0.109
LH×LR	4	0.782	0.552	0.778	0.554

GOIs		**psbA**		**psbD**		**psbC**		**PSBS**		**FD**		**RBCS**	
Effect	**df**	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***
Leaf Height	2	13.22	**0.000**	23.37	**0.000**	7.61	**0.004**	12.929	**0.000**	4.993	**0.019**	4.044	**0.035**
Leaf Rank	2	6.76	**0.006**	2.57	0.104	5.57	**0.013**	2.001	0.164	7.581	**0.004**	5.701	**0.012**
LH×LR	4	2.30	0.099	1.21	0.342	0.68	0.618	0.404	0.803	1.552	0.230	2.211	0.109

		CAB-151		**POR**		**HSP90**		**SHSP**		**AOX**		**BI**	
		F	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***
Leaf Height	2	4.032	**0.036**	9.825	**0.001**	0.369	0.697	0.86	0.438	22.4024	**0.000**	26.121	**0.000**
Leaf Rank	2	19.830	**0.000**	10.813	**0.001**	0.252	0.780	0.27	0.767	1.0733	0.363	2.307	0.128
LH×LR	4	1.750	0.183	0.858	0.507	1.675	0.200	1.84	0.165	2.3508	0.093	0.326	0.857

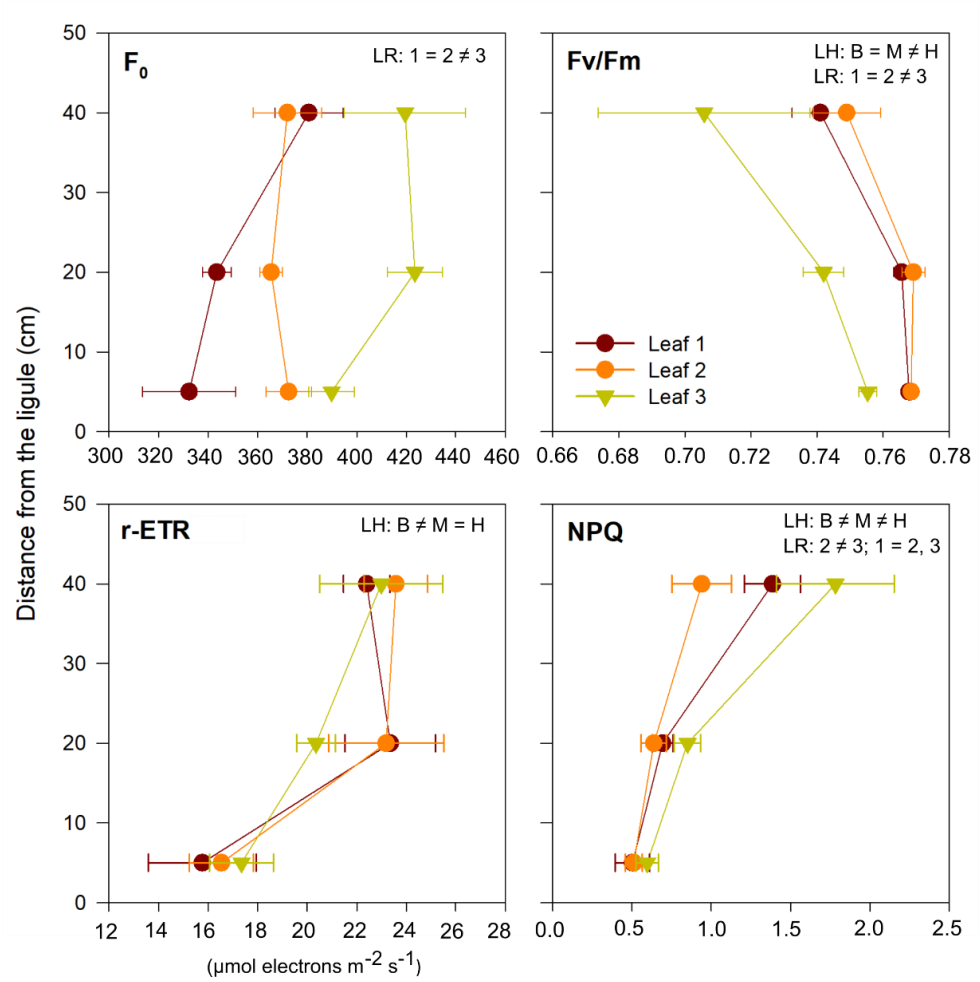

Fig. 2.5 Changes in photosynthetic parameters among and within *P. oceanica* leaves. F_0, basal fluorescence; Fv/Fm, maximum photochemical efficiency; r-ETR, relative electron transport rate; NPQ, non-photochemical quenching. Data are mean ± SE (*n*=3). Significant results of SNK post-hoc tests are reported on the top of the graphs.

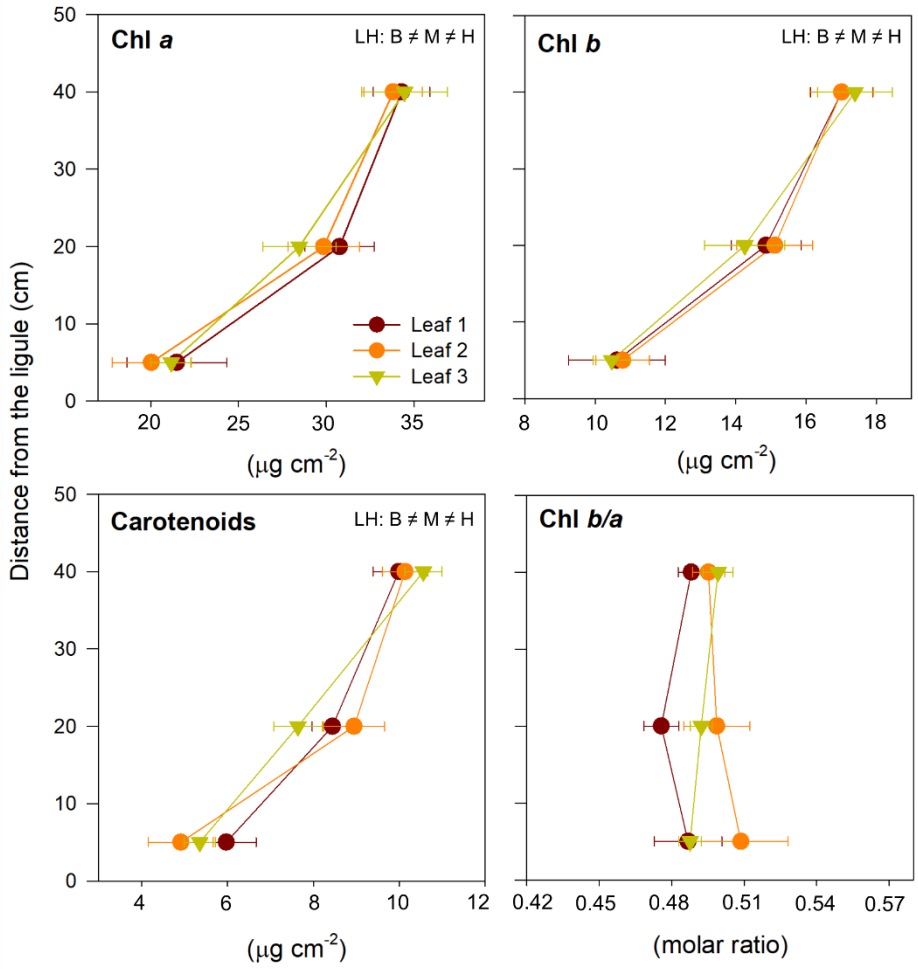

Fig. 2.6 Changes in chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and total carotenoids concentrations (in µg cm^{-2}), and Chl *b/a* molar ratio among and within *P. oceanica* leaves. Data are mean ± SE (*n*=3). Significant results of SNK post-hoc tests are reported on the top of the graphs.

Gene expression

The PERMANOVA revealed a significant effect of leaf height and leaf rank on the overall gene expression response ($P_{(perm)}$ <0.001; Table 2.5), with no interaction between the two factors. Subsequent post-hoc comparisons indicated significant differences in multivariate gene expression among all leaf sections established along the *P. oceanica* leaves (B *vs.* H, $P_{(MC)}$ <0.001; M *vs.* B and H, $P_{(MC)}$ <0.01). Among leaves, largest differences were observed between the youngest and mature ones (1 *vs.*3, $P_{(MC)}$ <0.01), and minor changes between rank 1 and 2 leaves (1 *vs.*2, $P_{(MC)}$ <0.05). No significant differences were found between leaves 2 and 3.

Similarly to what observed for the photo-physiology, the PCA of molecular data (Fig. 2.4B) emphasized a greater contribution of leaf height, more than leaf rank, in modulating the global gene-expression response. A substantial separation of three different sample groups was found along the PC1, which explains more than 60% of the total variance. The first group includes basal portions of leaves 1, 2, and 3 (on the left side of the plot), the second group includes middle portions of selected leaves (in the middle of the plot), and the third group comprises all the high-portion samples (on the right side of the plot). On the contrary, the PC2 (27 %) was related to leaf rank, as it separated the different leaves within the shoot, from the youngest to the mature. Interestingly, differences in the multivariate gene expression response were larger among basal portions of selected leaves than in medium and upper segments, where a more homogeneous pattern of expression was observed. As highlighted in the biplot, three genes related to photo-protection, cellular respiration and apoptotic processes (*PSBS*, *AOX* and *BI*) were the most positively correlated with the PC1, whereas genes contributing most to the PC2 were those involved in chlorophyll metabolism and photosynthesis (*CAB-151*, *FD*, *POR* and *RBCS*) (Table 2.6).

According to the two-way ANOVA, ten out of the 12 GOIs were significantly affected by one of the factor (leaf height and/or rank), but not from their interaction (Table 2.7). Only the two selected HSP proteins (*HSP90* and *SHSP*) did not alter their expression neither along nor among leaves. On the contrary, genes involved in all other targeted processes exhibited a differential regulation according to leaf developmental stages. As suggested by the PCA, LH had a stronger effect on gene expression. Ten target genes were significantly affected by LH, namely the subunits of PSII *psbA*, *psbD*, *psbC*, and *PSBS*, the photosynthetic electron carrier Ferredoxin (*FD*) and the RuBisCO enzyme (*RBCS*), together with chlorophyll-related genes *POR* and *CAB-151*, the Alternative oxidase 1a (*AOX*) and the apoptosis regulator Bax Inhibitor 1 (*BI*) (Table 2.7). Yet, genes with a significant differential expression among the different leaves of the shoot were only those involved in photosynthesis (*psbA, psbC, FD*

and *RBCS*), light harvesting proteins (*CAB-151*) and chlorophyll metabolism (*POR*) (Table 2.7).

Expression gradients of selected genes among and within *P. oceanica* leaves can be better appreciated from Fig. 2.7 and 2.8. Specifically, genes involved in photosynthesis and carbon fixation were generally more expressed in the youngest leaf compared to rank 2 and 3 leaves (Fig. 2.7), although differences were more pronounced in basal segments, respect to medium and upper sections (see details of SNK results on the top of the graphs). Along the leaf height, photosynthesis and photo-protection related-genes increased their expression from the base toward the apex (Fig. 2.7). A peculiar behavior was observed for the gene encoding for the RuBisCO (*RBCS*), as it exhibited the highest expression in the middle portion of rank 2 and 3 leaves, compared to basal and high segments, while the expression along the youngest leaf was more homogenous (Fig. 2.7). The opposite trend was detected for genes involved in chlorophyll metabolism (*POR*) and light harvesting (*CAB-151*). Their expression generally decreased from the basal to the upper portion of the leaf (Fig. 2.7). More importantly, for both genes there was a clear gradient of expression among the different leaves of the shoot, with a significant decrease in the expression from the youngest to the mature leaves (Fig. 2.7). The Alternative oxidase 1a (*AOX*) and *BI*, showed a clear gradient of expression along the longitudinal axis of selected leaves, increasing significantly their expression levels from the base toward the tip (Fig. 2.8). Although not significant, there was a trend for both genes to be more expressed in the third (mature) leaf, compared to younger ones, in sharp contrast to photosynthesis and chlorophyll metabolism-related genes.

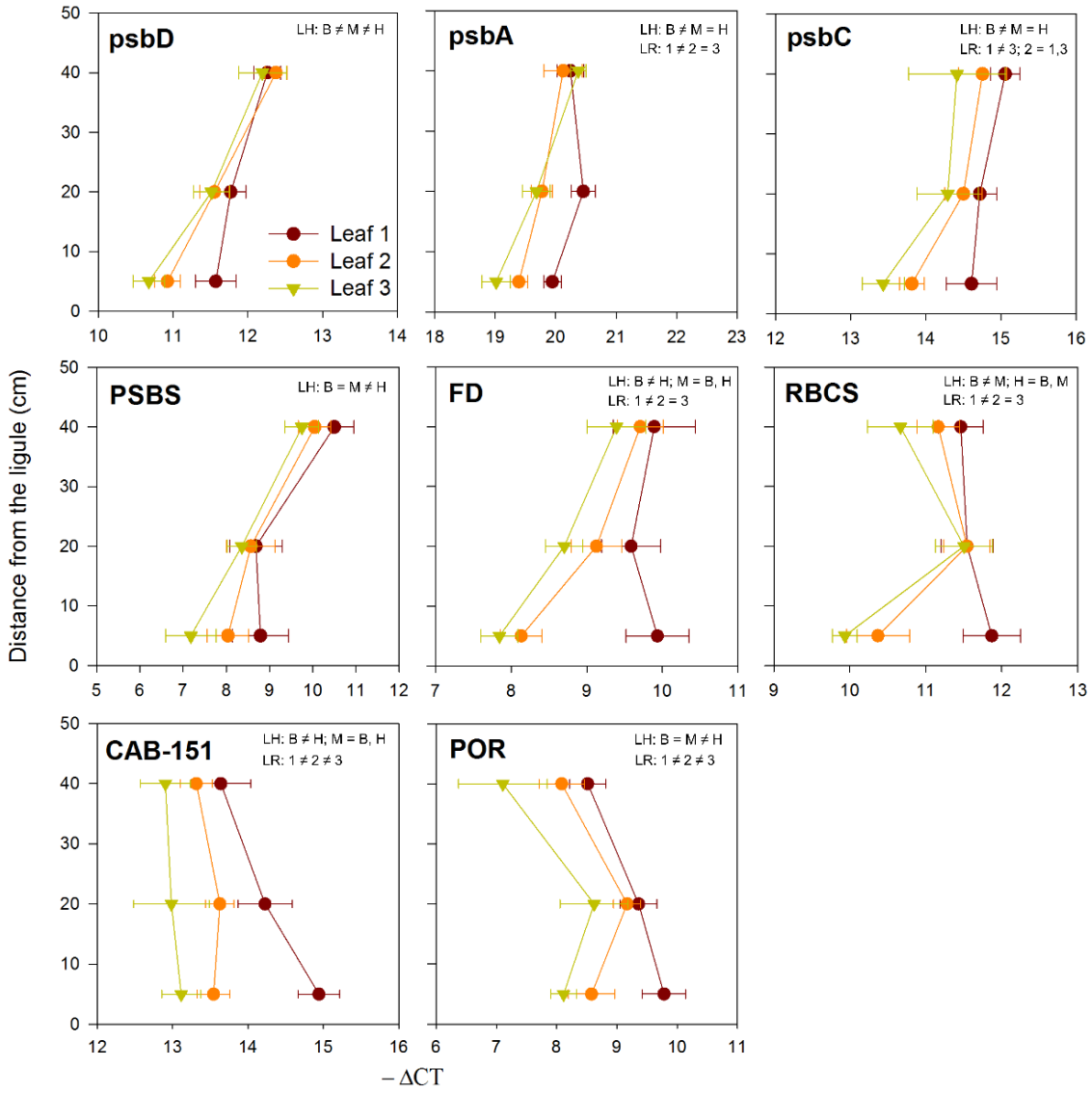

Fig. 2.7 Expression gradients (as -ΔCT) of genes related to photosynthesis, chlorophyll metabolism and carbon fixation among and within *P. oceanica* leaves. Data are mean ± SE (*n*=3). Higher -ΔCT values represent higher transcript accumulation. Significant results of SNK post-hoc tests are reported on the top of the graphs.

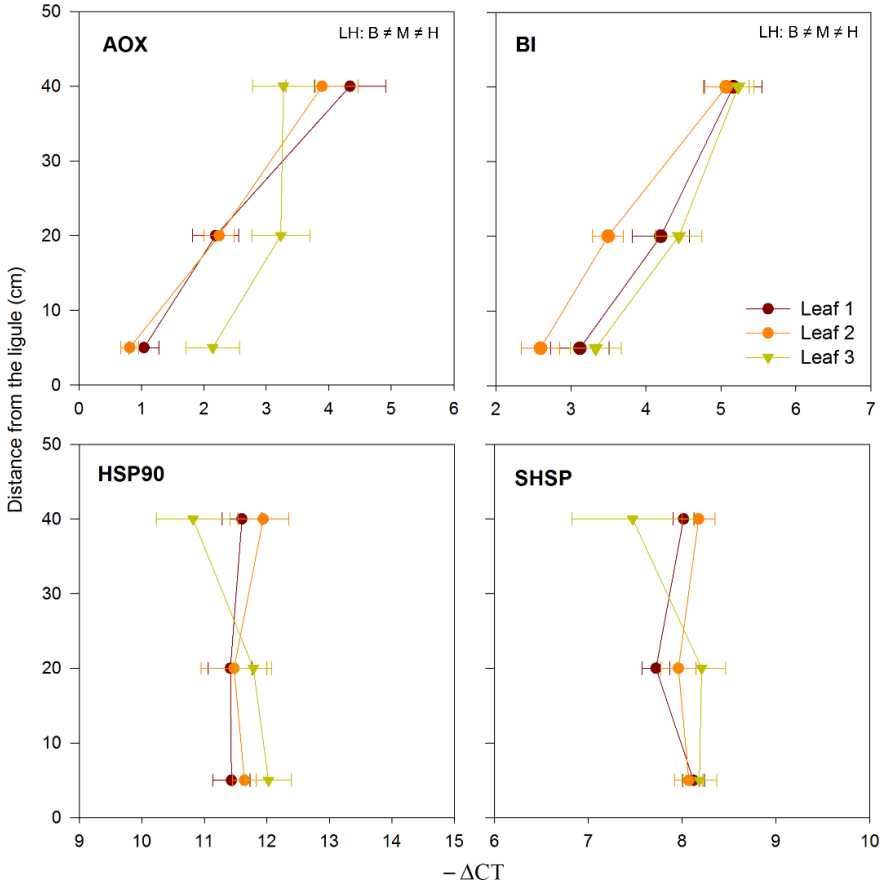

Fig. 2.8 Expression gradients (as -ΔCT) of genes involved in respiration, programmed cell death and heat shock proteins among and within *P. oceanica* leaves. Data are mean ± SE (*n*=3). Higher -ΔCT values represent higher transcript accumulation. Significant results of SNK post-hoc tests are reported on the top of the graphs.

Global DNA methylation

DNA methylation (5-mC) was analyzed only in the basal section of rank 1 leaf, middle section of rank leaf 2 and upper section of rank leaf 3, which represented the youngest, intermediate and oldest leaf tissues of all analyzed leaf segments. One-way ANOVA showed a significant effect of leaf developmental stages on global DNA methylation (P <0.05). Subsequent SNK post-hoc tests highlighted that the basal portion of leaf 1 contained significantly higher methylated DNA (5.7%) than medium section of leaf 2 (3%) and upper portion of leaf 3 (4.1%), however only comparisons between basal and middle sections were significant (P <0.05) (Fig. 2.9). Global DNA methylation levels of selected leaf segments did not show any significant correlation with overall gene expression (average expression of all GOIs) (Pearson's r =0.4952; P =0.175).

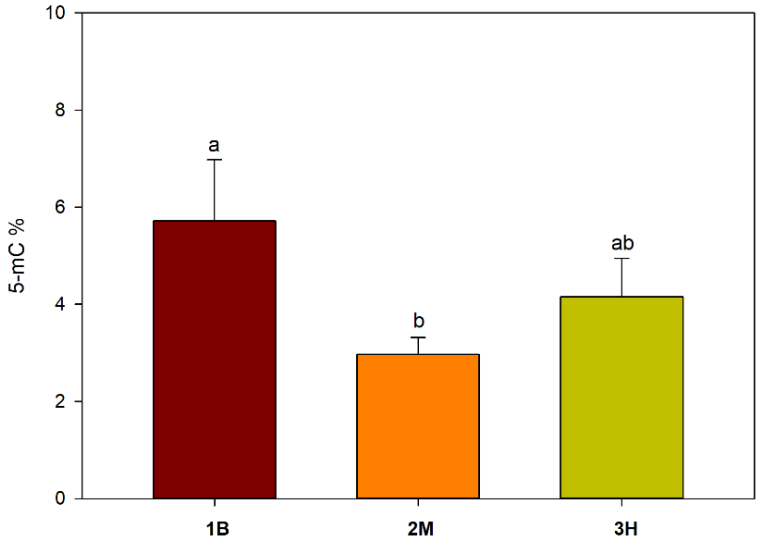

Fig. 2.9 Changes in DNA methylation (as % of 5-mC) in rank 1 leaf – basal, rank 2 leaf – medium and rank 3 leaf – high. Different letters indicate significant differences at *P* <0.05. Data are mean ± SD (*n*=3).

Correlations between photo-physiological and molecular data

Relationships between molecular and photo-physiological data were explored considering all targeted leaves together (rank 1, 2 and 3) (Fig. 2.10). Overall, transcript expression of PSII structural components (*psbA*, *psbD*, *psbC* and *PSBS*) was positively correlated with electron transport rate (r-ETR), pigment content (Chl*a*, Chl*b* and carotenoids) and NPQ, while a negative correlation was found with Fv/Fm (albeit significant only for *psbD*). Interestingly, also the Alternative oxidase (*AOX*) and Bax inhibitor (*BI*) genes followed a similar expression trend, resulting in a significant positive correlation with r-ETR, NPQ and pigments, and a marked negative correlation with Fv/Fm (Fig. 2.10). Chlorophyll-related genes *CAB-151* and *POR*, showed a significant negative correlation with Chl content and NPQ.

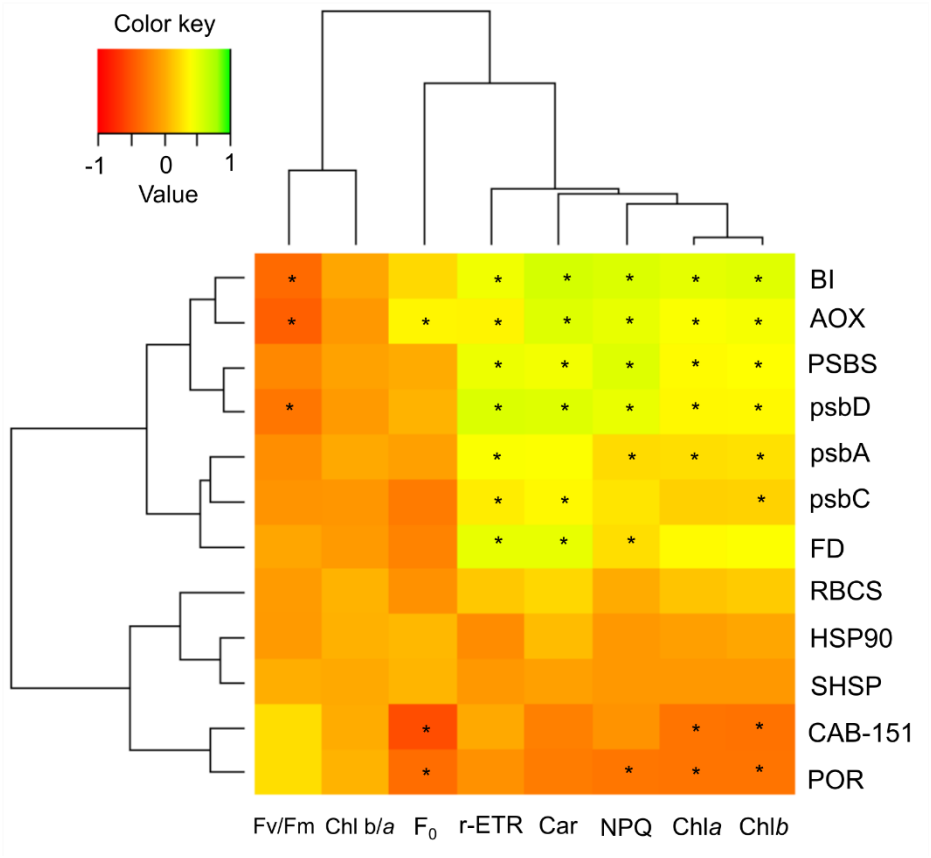

Fig. 2.10 Heatmap of Pearson's r coefficients depicting the relationships between individual gene expression and photo-physiological variables, considering the overall contribution of all rank leaves. Deeper colors indicate higher positive (green) or negative (red) correlations. Asterisks indicate significant correlations at *P* <0.05.

2.3.2 Within- and among-leaf variability in the heat stress response of P. oceanica

Morphology and fitness-related traits

Under heat stress, relative leaf growth rate of *P. oceanica* plants was 36 % lower than controls (*t*-test; P <0.05) (Fig. 2.11). Experimental treatment did not significantly affect leaf necrotic surface (*t*-test; P =0.07), although there was a tendency for plants exposed to 34°C to increase necrotized tissue (mean ± SE: control plants= 41.8 ±7.09, heated plants = 59.3 ±5.04 cm^2 $shoot^{-1}$).

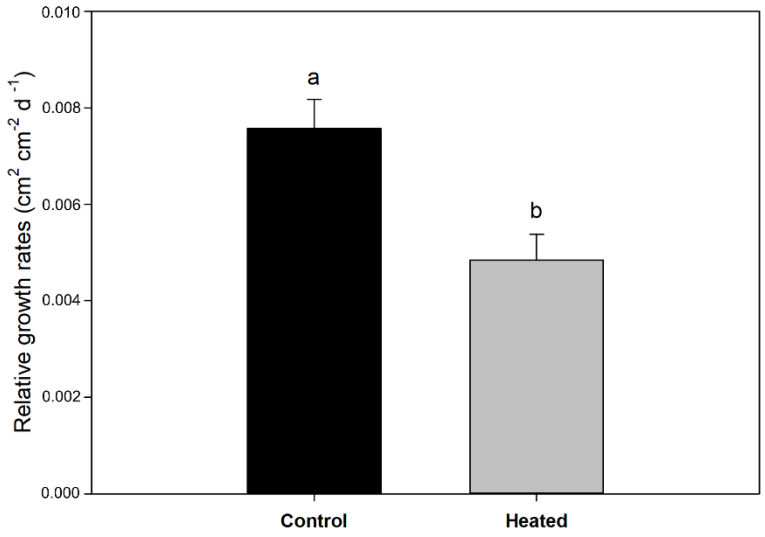

Fig. 2.11 Relative leaf growth rate (cm^2 cm^{-2} d^{-1}) in control and heated *P. oceanica* plants. Different letters indicate significant differences at P <0.05. Data are mean ± SE.

Photosynthetic parameters and pigment content

Multivariate analysis of photo-physiological variables (3-way PERMANOVA) highlighted the different role of distinct leaf portions in determining *P. oceanica* response to heat stress. Globally, photosynthetic parameters and pigment content were significantly affected either by the factor LH ($P_{(perm)}$ <0.001; Table 2.8) and treatment (heat) ($P_{(perm)}$ <0.001; Table 2.8) individually, and notably by their combination ($P_{(perm)}$ <0.01; Table 2.8). Multivariate pair-wise comparisons within each level of the factor height emphasized a significant effect of intense warming on basal and high leaf sections (control *vs.* heated in B: $P_{(MC)}$ <0.05 and H: $P_{(MC)}$ <0.001), in respect to middle segments (control *vs.* heated in M: $P_{(MC)}$ =0.07) (Table 2.8). Univariate 3-way ANOVA confirmed that all analyzed chlorophyll *a* fluorescence-derived photosynthetic parameters were affected by heat stress and varied according to LH

and/or LR, individually or in combination. Basal fluorescence (F_0) was significantly higher in plants exposed to 34°C only in basal (SNK, P <0.05) and middle leaf segments (SNK, P <0.001) (Table 2.9 and Fig. 2.12). Accordingly, maximum quantum yield of PSII (Fv/Fm) largely decreased under heat stress (P <0.001; Table 2.9 and Fig. 2.12) regardless the leaf rank and/or the leaf height. A similar pattern was observed for electron transport rate (r-ETR) which was greatly depressed in heated plants, in all sections established along the leaf length (SNK, P <0.001 for B, M and H; Table 2.9 and Fig. 2.12), without any difference with leaf rank. Non-photochemical quenching (NPQ) was also significantly affected by the treatment (P <0.001; Table 2.9). It increased under acute heat stress, particularly in distal leaf sections (Fig. 2.12).

Photosynthetic pigment content of *P. oceanica* (Chl*a*, Chl*b* and carotenoids) was affected by the interaction Heat×LH (Table 2.9). Specifically, both chlorophylls and carotenoids decreased significantly after heat stress only in uppermost leaf segments (SNK, P <0.001; Table 2.9 and Fig. 2.13). On the contrary, the antenna size (Chl *b*/*a*) was similar in control and heated plants, with a small decrease only in leaf 2 (SNK, P =0.09; Table 2.9 and Fig. 2.13).

Mean values (SE) of photosynthetic parameters and pigment concentrations determined in control and heated leaf samples are outlined in Table A2.1 in Appendix II.

Table 2.8 Results of 3-way PERMANOVAs conducted on photo-physiological variables (photosynthetic parameters and pigment content), and multivariate gene-expression data (-ΔCT values). $P_{(perm)}$ <0.05 are in bold, $P_{(perm)}$ <0.1 are underlined.

Three-way PERMANOVA

Photo-physiology

Source	**df**	**Pseudo-F**	$P_{(perm)}$	**Unique perms**	*Pair-wise tests*
Heat	1	33.360	**0.000**	9938	
Leaf Rank	2	1.017	0.376	9945	
Leaf Height	2	31.284	**0.000**	9945	LH: B ≠ M = H
Heat×LR	2	0.737	0.524	9951	
Heat×LH	2	7.411	**0.001**	9955	Basal: Control ≠ Heated Medium: Control = Heated (P =0.07) High: Control ≠ Heated
LR×LH	4	0.764	0.604	9942	
Heat×LR×LH	4	1.389	0.225	9932	

GOIs

Source	**df**	**Pseudo-F**	$P_{(perm)}$	**Unique perms**	*Pair-wise tests*
Heat	1	81.799	**0.000**	9945	
Leaf Rank	2	0.467	0.753	9959	
Leaf Height	2	3.168	**0.019**	9941	LH: B ≠ H; M = B, H
Heat×LR	2	0.986	0.392	9954	
Heat×LH	2	0.510	0.717	9946	
LR×LH	4	0.662	0.699	9940	
Heat×LR×LH	4	0.779	0.595	9938	

Table 2.9 Results of 3-way ANOVAs to assess the individual contribution of photo-physiological variables (photosynthetic parameters and pigment content) and GOIs. *P* <0.05 are in bold, *P* <0.1 are underlined.

Three-way ANOVA					
	Effect	**df**	**F**	***P***	**SNK pair-wise tests**
Photo-physiology					
F_0					
	Heat	1	22.692	**0.000**	
	Leaf Rank	2	24.530	**0.000**	1 ≠ 2 ≠ 3
	Leaf Height	2	3.056	0.059	
	Heat×LR	2	1.095	0.345	
	Heat×LH	2	4.612	**0.016**	Basal: Control ≠ Heated Medium: Control ≠ Heated High: Control = Heated
	LR×LH	4	1.602	0.195	
	Heat×LR×LH	4	1.695	0.173	
Fv/Fm					
	Heat	1	249.167	**0.000**	
	Leaf Rank	2	3.053	0.060	
	Leaf Height	2	14.808	**0.000**	B ≠ M = H
	Heat×LR	2	0.537	0.589	
	Heat×LH	2	1.722	0.193	
	LR×LH	4	0.145	0.964	
	Heat×LR×LH	4	0.868	0.493	
r-ETR					
	Heat	1	139.177	**0.000**	
	Leaf Rank	2	0.452	0.640	
	Leaf Height	2	9.423	**0.001**	B = M ≠ H
	Heat×LR	2	0.232	0.794	
	Heat×LH	2	4.953	**0.013**	Basal: Control ≠ Heated Medium: Control ≠ Heated High: Control ≠ Heated
	LR×LH	4	0.051	0.995	
	Heat×LR×LH	4	1.017	0.412	
NPQ					
	Heat	1	115.563	**0.000**	
	Leaf Rank	2	5.785	**0.007**	1 = 2 ≠ 3
	Leaf Height	2	76.159	**0.000**	B ≠ M ≠ H
	Heat×LR	2	0.305	0.739	
	Heat×LH	2	1.024	0.369	
	LR×LH	4	0.631	0.643	
	Heat×LR×LH	4	1.398	0.254	
Chl *a*					
	Heat	1	27.970	**0.000**	
	Leaf Rank	2	1.350	0.272	
	Leaf Height	2	29.880	**0.000**	B ≠ M = H
	Heat×LR	2	2.050	0.144	
	Heat×LH	2	7.810	**0.002**	Basal: Control = Heated Medium: Control = Heated High: Control ≠ Heated
	LR×LH	4	1.310	0.283	
	Heat×LR×LH	4	1.520	0.217	
Chl *b*					

Heat	1	28.712	**0.000**	
Leaf Rank	2	0.661	0.523	
Leaf Height	2	27.212	**0.000**	B ≠ M = H
Heat×LR	2	0.417	0.662	
				Basal: Control = Heated
Heat×LH	2	8.562	**0.001**	Medium: Control = Heated
				High: Control ≠ Heated
LR×LH	4	0.942	0.451	
Heat×LR×LH	4	1.666	0.179	
Carotenoids				
Heat	1	24.197	**0.000**	
Leaf Rank	2	1.864	0.170	
Leaf Height	2	44.328	**0.000**	B ≠ M ≠ H
Heat×LR	2	1.702	0.197	
				Basal: Control = Heated
Heat×LH	2	4.904	**0.013**	Medium: Control = Heated
				High: Control ≠ Heated
LR×LH	4	0.372	0.827	
Heat×LR×LH	4	0.666	0.620	
Chl *b/a*				
Heat	1	0.338	0.564	
Leaf Rank	2	1.560	0.224	
Leaf Height	2	0.007	0.993	
				1: Control = Heated
Heat×LR		4.255	**0.022**	2: Control ≠ Heated (*P* =0.09)
	2			3: Control = Heated
Heat×LH	2	1.594	0.217	
LR×LH	4	0.778	0.547	
Heat×LR×LH	4	0.890	0.480	
GOIs				
psbA				
Heat	1	3.932	0.055	
Leaf Rank	2	2.006	0.149	
Leaf Height	2	14.872	**0.000**	B ≠ M = H
				1: Control ≠ Heated
Heat×LR	2	3.042	0.060	2: Control = Heated
				3: Control = Heated
Heat×LH	2	1.694	0.198	
LR×LH	4	0.503	0.733	
Heat×LR×LH	4	2.220	0.086	
psbD				
Heat	1	0.118	0.733	
Leaf Rank	2	0.269	0.766	
Leaf Height	2	9.273	**0.001**	B ≠ M = H
Heat×LR	2	0.631	0.538	
Heat×LH	2	2.405	0.105	
LR×LH	4	1.544	0.210	
Heat×LR×LH	4	1.583	0.200	
psbC				
Heat	1	0.860	0.360	
Leaf Rank	2	0.233	0.793	
Leaf Height	2	1.797	0.180	
Heat×LR	2	1.233	0.303	
Heat×LH	2	0.106	0.900	
LR×LH	4	1.028	0.406	
Heat×LR×LH	4	0.836	0.511	
PSBS				

	Heat	1	33.994	**0.000**	
	Leaf Rank	2	0.274	0.762	
	Leaf Height	2	13.667	**0.000**	B = M ≠ H
	Heat×LR	2	1.271	0.293	
	Heat×LH	2	0.439	0.648	
	LR×LH	4	0.542	0.706	
	Heat×LR×LH	4	0.380	0.822	
FD					
	Heat	1	77.251	**0.000**	
	Leaf Rank	2	0.276	0.760	
	Leaf Height	2	0.701	0.503	
	Heat×LR	2	1.844	0.173	
	Heat×LH	2	0.115	0.892	
	LR×LH	4	0.465	0.761	
	Heat×LR×LH	4	0.892	0.479	
RBCS					
	Heat	1	70.165	**0.000**	
	Leaf Rank	2	1.491	0.231	
	Leaf Height	2	0.606	0.548	
	Heat×LR	2	0.954	0.389	
	Heat×LH	2	0.244	0.784	
	LR×LH	4	0.563	0.690	
	Heat×LR×LH	4	1.037	0.393	
CAB-151					
	Heat	1	161.684	**0.000**	
	Leaf Rank	2	1.686	0.200	
	Leaf Height	2	1.007	0.375	
	Heat×LR	2	1.484	0.240	
	Heat×LH	2	0.205	0.816	
	LR×LH	4	0.572	0.685	
	Heat×LR×LH	4	1.212	0.323	
POR					
	Heat	1	59.937	**0.000**	
	Leaf Rank	2	2.199	0.126	
	Leaf Height	2	2.532	0.094	
	Heat×LR	2	1.734	0.191	
	Heat×LH	2	0.801	0.457	
	LR×LH	4	0.600	0.665	
	Heat×LR×LH	4	0.655	0.627	
HSP90					
	Heat	1	9.036	**0.005**	
	Leaf Rank	2	1.414	0.256	
	Leaf Height	2	1.919	0.162	
	Heat×LR	2	1.166	0.323	
	Heat×LH	2	0.087	0.917	
	LR×LH	4	0.823	0.519	
	Heat×LR×LH	4	1.377	0.261	
SHSP					
	Heat	1	0.732	0.398	
	Leaf Rank	2	0.120	0.887	
	Leaf Height	2	1.108	0.341	
	Heat×LR	2	0.126	0.882	
	Heat×LH	2	0.029	0.972	
	LR×LH	4	0.237	0.915	
	Heat×LR×LH	4	0.157	0.959	
AOX					
	Heat	1	424.021	**0.000**	

	Leaf Rank	2	0.909	0.412	
	Leaf Height	2	16.731	**0.000**	B ≠ M ≠ H
	Heat×LR	2	0.209	0.812	
	Heat×LH	2	4.518	**0.018**	Basal: Control ≠ Heated Medium: Control ≠ Heated High: Control ≠ Heated
	LR×LH	4	0.837	0.511	
	Heat×LR×LH	4	1.707	0.170	
BI					
	Heat	1	287.155	**0.000**	B = M ≠ H
	Leaf Rank	2	1.479	0.241	
	Leaf Height	2	8.133	**0.001**	
	Heat×LR	2	0.635	0.536	
	Heat×LH	2	5.213	**0.010**	Basal: Control ≠ Heated Medium: Control ≠ Heated High: Control ≠ Heated
	LR×LH	4	0.626	0.647	
	Heat×LR×LH	4	0.401	0.807	

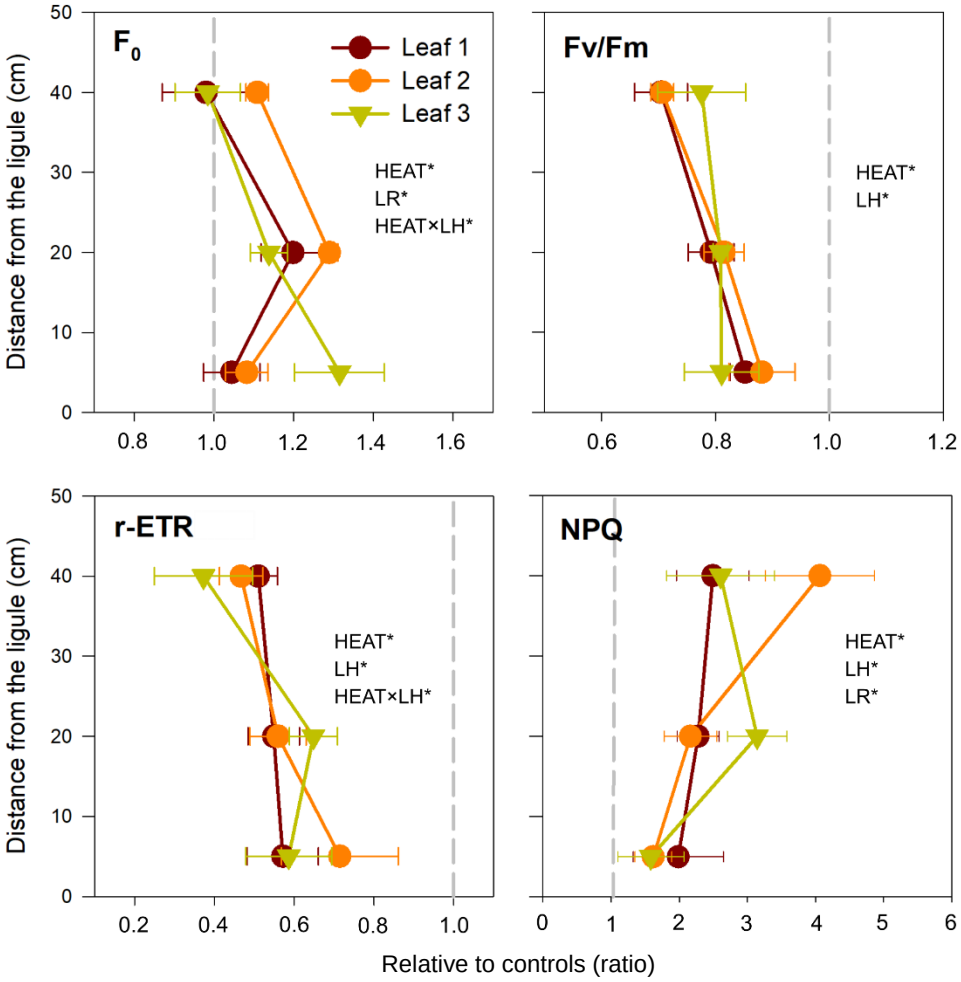

Fig. 2.12 Changes in photosynthetic parameters, in heated relative to control plants (ratio). F_0, basal fluorescence; Fv/Fm, maximum photochemical efficiency of PSII; r-ETR, relative electron transport rate; NPQ, non-photochemical quenching. Values on the left or right of the dashed grey line represent a decrease or increase in respect to controls, respectively. Data are mean ± SE (*n*=3). Significant results of 3-way ANOVA are reported on the top of the graphs. For details of SNK post-hoc tests see Table 2.9.

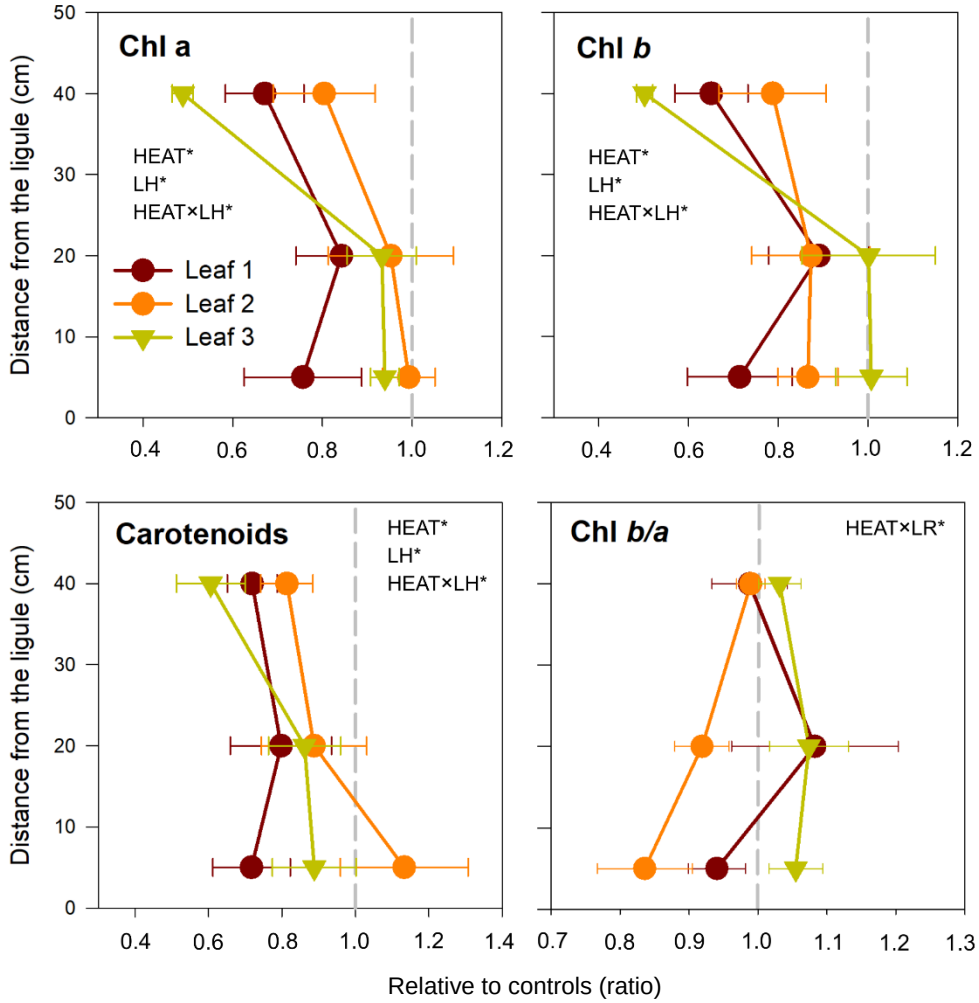

Fig. 2.13 Changes in chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), total carotenoids concentrations and Chl *b/a*, in heated relative to control plants (ratio). Values on the left or right of the dashed grey line represent a decrease or increase in respect to controls, respectively. Data are mean ± SE (*n*=3). Significant results of 3-way ANOVA are reported on the top of the graphs. For details of SNK post-hoc tests see Table 2.9.

Gene expression

Three-way PERMANOVA confirmed a strong significant effect of acute heat stress ($P_{(perm)}$ <0.001; Table 2.8) and leaf height ($P_{(perm)}$ <0.05; Table 2.8) on multivariate gene-expression response of *P. oceanica*, with no interaction between the two factors.

On the other hand, analysis of individual gene expression by means of univariate 3-way ANOVA, revealed that such interaction was restricted to genes involved in respiration and plant PCD (*AOX* and *BI*). Most of targeted genes (9 out of 12 GOIs) were significantly affected by intense warming, regardless the leaf rank and/or height, for example those involved in photosynthesis, light harvesting/chlorophyll biosynthesis and carbon assimilation pathways (Table 2.9).

Core photosystem II subunits (*psbD* and *psbA*) exhibited a peculiar behavior, with a general pattern of down-regulation in the youngest leaf (rank 1), whereas in the leaves 2 and 3 they were either slightly up or down-regulated depending on the leaf section considered (Fig. 2.14). Only for *psbA*, the response to heat stress varied significantly according to leaf rank (SNK, control *vs.* heated in leaf 1: P <0.05; Table 2.9).

Genes involved in non-photochemical quenching (*PSBS*), photosynthetic electron transport (*FD*) and Calvin cycle (*RBCS*) were all negatively affected by acute warming. They were significantly down-expressed in all established leaf sections (P <0.001; Table 2.9), with strongest values recorded in youngest leaf tissues (basal and middle portions of leaf 1), where e.g. *RBCS* and *FD* were down-regulated up to 60 and 100 fold changes, respectively, when compared to control plants (Fig. 2.14). A quite similar behavior was shown by chlorophyll-related genes (light harvesting and chlorophyll biosynthesis) (*CAB-151* and *POR*), which were significantly negatively affected by heat stress (P <0.001; Table 2.9), again at higher level in basal and middle sections of leaf 1 (Fig. 2.14).

Among the two selected HSP proteins (*HSP90* and *SHSP*), only *HSP90* was significantly over-expressed in heated plants with respect to controls (P <0.01; Table 2.9), with highest values in the basal section of leaf 1 and upper portion of leaf 3 (Fig. 2.15). *AOX* and *BI* were the two most up-regulated genes under heat stress of the whole dataset (P <0.001; Table 2.9). They were significantly induced in all sections selected along the leaf length (SNK, control *vs.* heated in B, M and H: P <0.001; Table 2.9), particularly in basal segments of leaves 1 and 2, where they reached expression values up to 90 fold changes higher than control plants (Fig. 2.15).

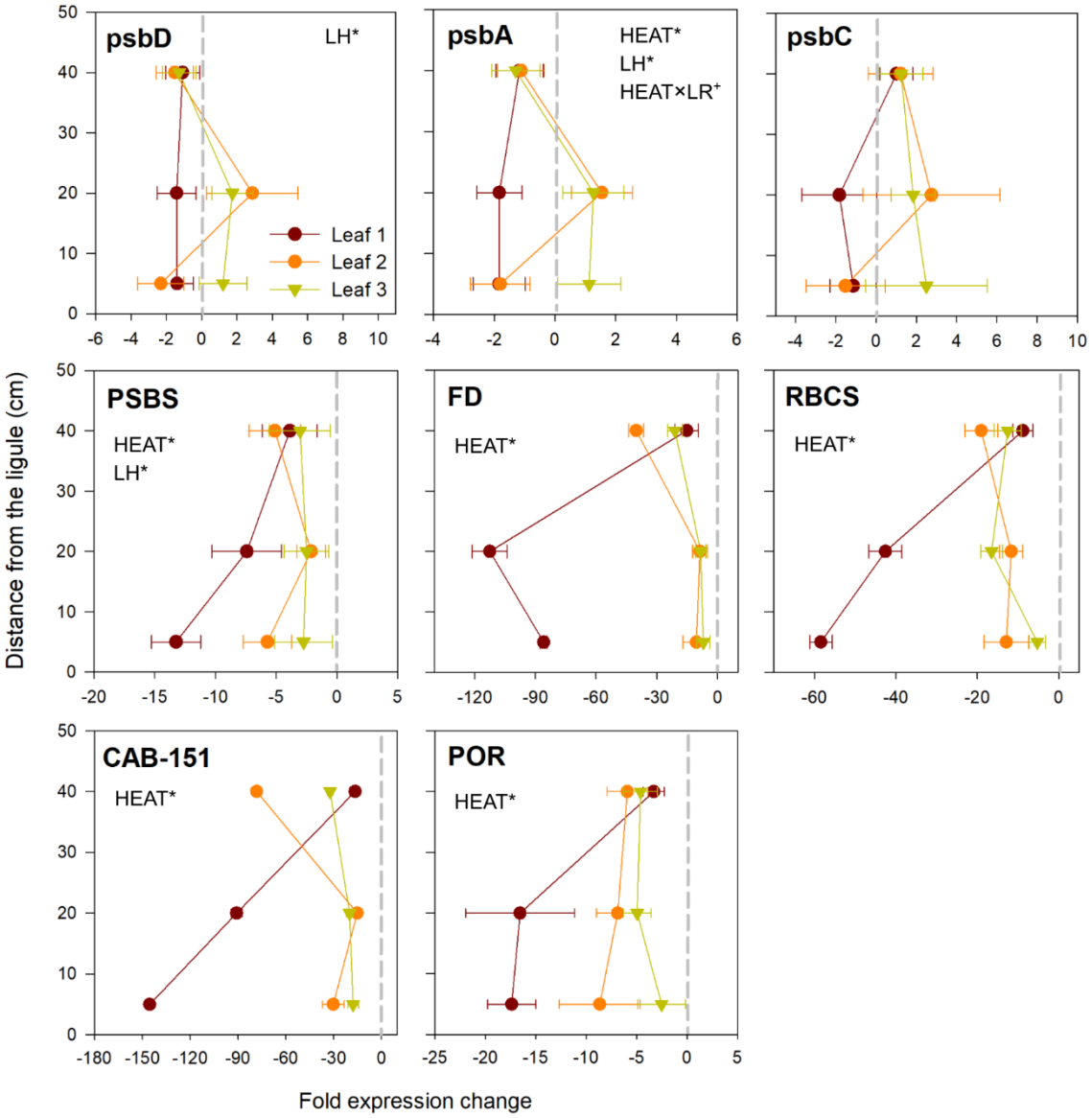

Fig. 2.14 Relative expression of genes involved in photosynthesis, chlorophyll metabolism and carbon fixation in heated *vs.* control plants. Data are mean ± SE (*n*=3). Negative fold changes represent transcript down-regulation and vice versa. Significant results of 3-way ANOVA are reported on the top of the graphs. For details of SNK post-hoc tests see Table 2.9.

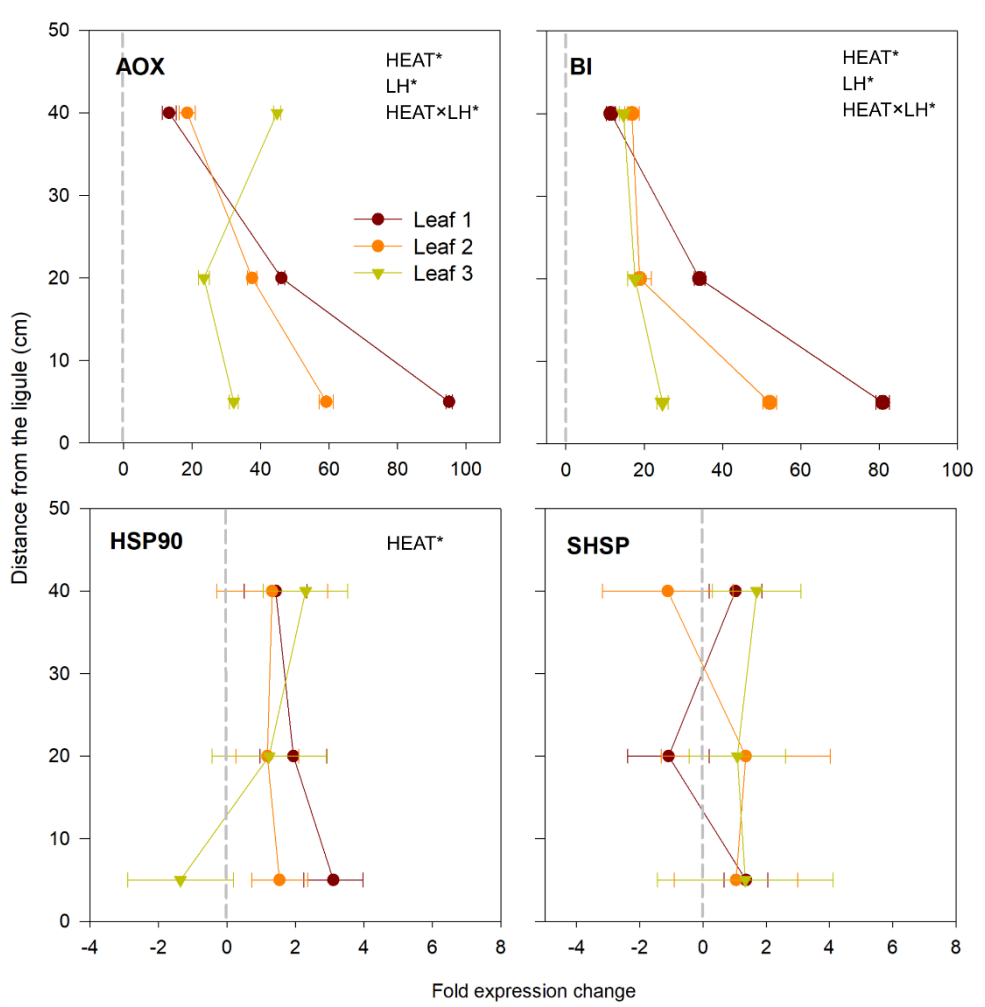

Fig. 2.15 Relative expression of genes involved in respiration, programmed cell death and heat-shock proteins in heated *vs.* control plants. Data are mean ± SE (*n*=3). Negative fold changes represent transcript down-regulation and vice versa. Significant results of 3-way ANOVA are reported on the top of the graphs. For details of SNK post-hoc tests see Table 2.9.

Global DNA methylation

Two-way ANOVA highlighted a significant effect of intense warming on global DNA methylation (P <0.05), without a significant leaf-age effect. Only in intermediate (2M) and oldest leaf portions (3M), heat stress-induced increase in % of methylated DNA was evident, whereas youngest leaf segments (1B) had a comparable level of methylated DNA in heated and control conditions (Fig. 2.16). Pairwise comparisons between heated and control samples for 2M and 3H leaf segments were close to the significance level (*t*-test; 2M: control *vs.* heated P =0.07; 3H: control *vs.* heated P =0.10).

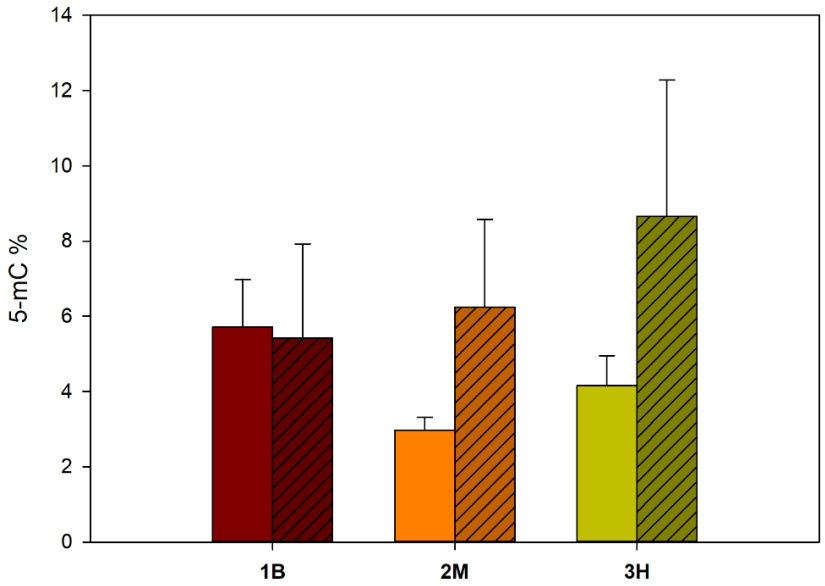

Fig. 2.16 Changes in DNA methylation (as % of 5-mC) in rank 1 leaf – basal, rank 2 leaf – medium and rank 3 leaf – high, under control and heated conditions (dashed bars). Data are mean ± SD (*n*=3).

2.4 Discussion

2.4.1 *The interplay between irradiance and developmental cues modulates photo-physiological and gene-expression patterns among and within P. oceanica leaves*

A photo-physiological and gene expression survey was performed at three heights established along the longitudinal axis of three *P. oceanica* leaves, to capture the natural variation in physiological and molecular functions along the two age gradients existing within the seagrass shoot: i.e. from the inner to the outer leaves, and from the basis to the apex of each leaf. Yet, for the first time, the variation in global DNA methylation level (5-mC) according to leaf-tissue age was explored in *P. oceanica*.

Although age-dependent variations in leaf photosynthetic performance and pigment content have been previously described for several seagrass species, including *P. oceanica*, here I provide first evidence that such variations in photo-physiological functions can be related with localized transitions in mRNA abundance of specific genes involved in these processes. Global DNA methylation was shown to vary with leaf age, likely due to the interplay between developmental and light cues.

Photo-physiological and molecular multivariate results converged in suggesting that the vertical gradient existing along the leaf blade of *P. oceanica*, from the base toward the tip, was much stronger than the horizontal one established among the different leaves of the shoot. However, when comparing the youngest leaf, with young and mature leaves of the shoot, a significant change in the global gene expression response was detected, but without a corresponding change in photo-physiology. On the other hand, univariate analyses showed that, at least for some photosynthetic parameters (i.e. F_0 and Fv/Fm), a leaf-rank gradient was clearly present too.

The observed within- and among-leaves physiological and molecular variations were related to both age and irradiance levels, though the relative contribution of each of these two factors seems to vary depending on the specific gradient considered. The former one (within leaves) being mainly accounted for by the strong vertical irradiance gradient present within *Posidonia* meadows, while the later one (among leaves) reflecting mainly age differences among leaf tissues.

Changes in photosynthetic performance and pigment content from the base toward the leaf tip can be particularly relevant in large-sized subtidal seagrass species (e.g. *Posidonia* and *Thalassia* spp.), due to the strong self-shading caused by dense canopies formed by these species (Alcoverro et al. 1998; Dalla Via et al. 1998; Durako and Kunzelman 2002; Enríquez

et al. 2002; Ralph et al. 2007). Contrasting results have been found for smaller-sized species (e.g. in *Z. marina*; Ralph et al. 2002), more likely because of the lower structural complexity of their canopies, leading to a more homogenous light environment along the leaves.

In the large-sized species *P. australis*, Ralph and Gademann (1999) evidenced that the photochemical capacity of leaves (Fv/Fm) decreased from the base towards the distal end of the leaf blade. Similarly, in the tropical *T. testudinum*, the Fv/Fm ratio of the second rank leaf was found to decline at an average rate of 1.5% cm^{-1} toward the tip (Enríquez et al. 2002). Results presented here for *P. oceanica* nicely fit this model as, in all selected leaves, Fv/Fm declined progressively from the 5 to the 40 cm leaf sections, with similar values detected between basal and middle segments, and strong changes in the upper sections. This strong reduction in photochemical efficiency between medium and high leaf segments, but weak between basal and medium portions, can be mainly related to the strong light attenuation gradient existing within the *P. oceanica* meadows. In fact, a sharp light reduction occurs in the upper centimetres of the canopy and then light decreases more softly down to the meadow bottom, where light intensity can be less than 3% of the subsurface irradiance (Dalla Via et al 1998; Marìn-Guirao et al. 2015). This means that basal leaf sections are almost completely shaded, whereas upper leaf portions are progressively exposed to higher and even damaging irradiance levels.

Due to this strong light gradient, upper leaf portions experience some degree of photo-damage, as reflected here by their higher basal fluorescence (F0) and decrease in photochemical efficiency (Fv/Fm) (Major and Dunton 2002; Enrìquez et al. 2002). At molecular level, the expression of photosynthetic PSII reaction center genes (*psbA* and *psbD*), showed a negative correlation with Fv/Fm (albeit only significant for *psbD*), with increasing transcript accumulation from the base to the upper leaf portions. This supports the presence of a chronic photo-inhibition in distal leaf tissues, caused largely by photo-damage to core proteins of PSII necessitating their replacement (Aro et al. 1993; Mulo et al. 2012).

The decline in photochemical efficiency (Fv/Fm) paralleled the increase in non-photochemical quenching (NPQ) along the leaf blade (toward the distal end of the leaf). NPQ harmlessly quenches the excitation of chlorophyll within the light-harvesting antennae of PSII by converting excitation energy into thermal energy that can be released (Ruban 2016). Therefore, these results confirm the activation of photo-protective mechanisms and the existence of a strong vertical irradiance gradient. The same trend was observed for the *PSBS* gene, which plays a fundamental role in NPQ (Niyogi et al. 2005). Larger capacity for thermal energy dissipation toward the leaf tip was also observed in *T. testudinum* for the

second rank leaf, and was positively associated with increase in xanthophyll cycle pigment content and in the proportion of Zeaxanthin in the total xanthophyll pool (VAZ/Chl *a*) (Schubert et al. 2015).

Interestingly, also the transcript for the Alternative oxidase 1 enzyme (*AOX*) showed the same trend of *PSBS*, increasing its abundance from the young leaf base to the higher leaf sections, and was positively correlated with NPQ. Aox is one of the two terminal oxidases in the mitochondrial respiratory chain, and catalyzes the cyanide-resistant oxidation of ubiquinol and the reduction of molecular oxygen to water. Contrarily to the cytochrome oxidase (Cox) pathway, the mitochondrial alternative oxidase pathway is uncoupled from proton translocation, thus bypassing ATP production, and heat is generated instead (Finnegan et al. 2004). Among the respiratory-related genes, *AOX* is the component showing a clear light-dependent up-regulation at transcript and protein levels (Svensson and Rasmusson 2001; Yoshida and Noguchi 2009), and this has been demonstrated also in seagrasses (Procaccini et al. 2017). To date, multiple physiological roles of Aox have been revealed in terrestrial higher plants (Clifton et al. 2006; Xu et al. 2011). In mitochondria, it is known to play a key role in the maintenance of mETC redox homeostasis and the regulation of ascorbate biosynthesis (Vishwakarma et al. 2015). In addition, the importance of Aox in dissipating excess chloroplast reducing equivalents to optimize and protect photosynthesis from photo-inhibition or prevent photo-oxidative stress, has been clearly demonstrated in the past two decades (Zhang et al. 2011; Zhang et al. 2012b; Vishwakarma et al. 2014). More recently, the involvement of Aox in the NPQ induction was established in *Arabidopsis* (Vishwakarma et al. 2015). This suggests that the adjustment of the mitochondrial Aox pathway, and the underlying cross-talk between mitochondria and chloroplasts, can be one of the key driver of the photoacclimatory capacity observed along seagrass leaves. Alike *PSBS* and *AOX*, Bax Inhibitor-1 (*BI*) was one of the genes contributing most to the separation of leaf height groups in the PCA, and exhibited a significant variation along the *P. oceanica* leaf, with a similar pattern of aforementioned genes. *BI* is a conserved cell death suppressor in both animals and plants (Watanabe and Lam 2006), whose expression level is generally enhanced during senescence and under several types of biotic and abiotic stresses (Watanabe and Lam 2006). In plants, programmed cell death (PCD) is a genetically controlled process, and ROS have been proposed as key inducers of different types of developmental and/or environmental PCD (De Pinto et al. 2012). The mitochondrion, in particular, has been shown to be involved in ROS-mediated PCD (Amirsadeghi et al. 2006; Petrov et al. 2015). The induction of the Aox pathway, dampening ROS formation, seems to act as an important mechanism to prevent the activation of such

PCD pathways responsive to mitochondrial respiratory status (Robson and Vanlerberghe 2002; Vanlerberghe et al. 2002). Therefore, the hypothesis is that ROS accumulation along seagrass leaves, due to the interplay between senescence processes due to the high-light exposure of upper leaf sections, might co-induce *BI* and *AOX* gene expression, that would act both as PCD suppressors through direct (*BI*) and indirect (*AOX*) mechanisms.

The photosynthetic electron transport rate (r-ETR) along *P. oceanica* leaves, showed the highest values in middle and upper leaf segments, compared to the base, similarly to what was found by Ralph and Gademann (1999) in *P. australis* for the second rank leaf, reflecting the higher photosynthetic performance of these leaf portions in marine plants. The transcript for the chloroplast electron carrier Ferredoxin (*FD*) exhibited a similar expression trend along the leaf length. Ferredoxin is indeed a key component of the chloroplast ETC that plays an important role in the final step of the linear electron flow, thanks to its ability to divert electrons to cyclic or alternative electron flow pathways, sustaining photosynthesis and minimizing damaging ROS production (Munekage et al. 2004). The gene encoding for the small subunit of the RuBisCO enzyme (*RBCS*) showed a peculiar behavior, being expressed at almost constant level in the leaf 1, while peaking in the middle and high sections of leaves 2 and 3. This agree with previous records of maximum photosynthetic rate in the middle portions of intermediate leaves, indicating an optimum use of light in such tissues and evidencing the key importance of this shoot portion as energy source for the whole plant (Mazzella and Alberte 1986; Alcoverro et al. 1998; Enríquez et al. 2002; Olivé et al. 2013).

Photosynthetic pigment content of *P. oceanica* (Chl*a*, Chl*b* and carotenoids) varied along the leaf length. Basal leaf sections contained significantly less chlorophylls and carotenoids than middle and upper segments, likely due to their young/immature age, since light conditions were similar to middle portions, as commented above. These results seem to contrast with previous reports in seagrasses, where photosynthetic pigment content was found to increase from the base to the middle leaf sections, then decreasing toward the tip, which exhibited the lowest Chl content (Dalla Via et al. 1998; Enrìquez et al. 2002; Olivè et al. 2013). This discrepancy can be explained considering that the highest leaf sections (at 40 cm from the ligule) selected for this work did not correspond to the leaf tip in leaves 2 and 3.

Chlorophyll-related genes (*CAB-151* and *POR*) showed a quite opposite behavior respect to pigment content, with increased abundance in base and middle segments, compared to the leaf apex (although for *CAB-151* this was only noticeable in the leaf 1). This could reflect the maturation of young tissues that progressively acquire full photosynthetic competency Therefore, the up-regulation of the molecular machinery responsible for producing high

level of enzymes involved in the tetrapyrrole biosynthesis and light harvesting proteins occurs in basal/middle leaf sections, then slowing down toward the leaf tip, where such complexes were already at high level.

As commented above, pho-physiological variations were less intense among leaves than within leaves and seem to respond mainly to differences in leaf age, although the influence of irradiance becomes progressively more important toward the leaf tip. In addition to photochemical reduction observed from the bottom to the leaf apex, a reduction from the inner to the outer leaves was also observed. The mature leaf showed significantly lower Fv/Fm values than younger leaves (1 and 2), similarly to what found in *T. testudinum* by Durako and Kunzelman (2002). These differences were stronger in the upper leaf segments of leaf 3, where an acute increase in basal fluorescence (F0) also occurred. Since the position of this leaf within the shoot is comparable to that of leaf 2, the light environment they experience can be considered quite similar; therefore, the observed differences are more likely due to the longer life history exposure of these segments to high irradiance levels, as they represent the oldest tissues analyzed in this study. Indeed, NPQ was similar between the younger (inner leaf 1) and older (leaf 3) analyzed leaves, and differences were only found between upper leaf segments. Moreover, genes participating in leaf tissue photo-acclimation (e.g. *PSBS*, *AOX* and *BI*), neither showed differences among leaves, besides the large variability along the leaf blade. The notion that the physiological variation among leaves is mainly related to leaf age is supported also by the fact that most of analyzed genes encoding for key photosynthetic structural proteins and enzymes showed a similar expression pattern, with higher transcript accumulation in younger leaves and lower in older ones. In general, these inter-leaf differences were stronger at the leaves base, where changes in irradiance level should be minimal. The intense production of functional and structural photosynthetic proteins in younger leaves in comparison to old ones reflects leaf tissue maturation, for which high amounts of proteins has to be synthetized and assembled to allow the leaf tissue to acquire full photosynthetic competency (Li et al. 2010; Mattiello et al. 2015). Moreover, these basal leaf tissues showed the lowest photosynthetic (i.e. Fv/Fm, r-ETR and NPQ) variability, in accordance with their similar light environment.

Global DNA methylation analysis in *P. oceanica* revealed a significant variation depending on leaf developmental stages, with higher % methylation recorded in the youngest and oldest analyzed tissues (1B and 3H), and lowest values in intermediate tissues (2M). This indicates that a high level of methylation is present in younger cells, which decreases progressively in mature leaf tissues, while increasing again with leaf ageing.

As discussed above, young and basal tissues need to activate a high number of genes to allow tissue maturation, which are no longer needed in intermediate leaf portions exposed to non-damaging light levels, whereas older distal tissues require the activation of specific sets of genes for their successful photo-acclimation and photo-protection to high irradiance levels. Accordingly, a moderate (non-significant), positive correlation between DNA methylation and overall gene expression was observed.

DNA methylation and gene transcription are closely interwoven processes (Zilberman et al. 2006), however the significance of such methylation and its relation with gene activation/ suppression is strongly dependent upon the underlying sequence and its location in the plant genome (e.g. promoters *vs.* transcribed regions) (Niederhuth and Schmitz 2017). For example, methylation of transposable elements and promoter region of a gene generally leads to silencing (Li et al. 2012), whereas methylation inside gene bodies has shown to positive regulate gene expression (Lu et al. 2015). Although these results points for a hyper-methylation in more transcriptionally active and high-light exposed tissues, this should be taken with caution, due to the small number of genes considered and the difficulty to correlate a global DNA methylation analysis with the expression of individual genes.

Epigenetic research in seagrasses is still at the infancy, and the lack of genomic resources for most species, including *P. oceanica*, makes it hard to conduct detailed studies that could contribute to a more mechanistic understanding of the role of DNA methylation and other epigenetic modifications in the regulation of gene expression. However, these results represent a useful starting point for future research on this topic, suggesting that epigenetic modifications do occur during seagrass leaf development and could affect the expression of genes responsible for leaf maturation, acquisition of photosynthetic competence and light acclimation (Greco et al. 2013).

2.4.2 Differential leaf age-dependent thermo-tolerance in P. oceanica

Results presented here highlighted once more the strong negative effect of acute short-term heat stress on *P. oceanica*, in terms of photo-physiological and gene-expression alterations underlying responses at higher level of organization (e.g. growth). Notably, the temperature of 34°C to which plants were exposed for one week is the highest among the ones chosen for previous mesocosm studies in *P. oceanica*, where many recent reports selected a maximum exposure temperature of 32°C (Olsen et al. 2012; Marín-Guirao et al. 2016; Marín-Guirao et al. 2017; Tutar et al. 2017; Traboni et al. 2018).

The analysis was conducted at fine spatial resolution and allowed to establish that the response to heat stress in *P. oceanica* varied with leaf developmental stages. As expected from results obtained in the previous section, the age gradient existing along the longitudinal axis of *P. oceanica* leaves, from the base toward the tip, seems to affect photo-physiological responses to heat stress more than leaf rank-differences. At gene-expression level, youngest sections of rank leaf 1 (B and M), exhibited the strongest negative response to warming, suggesting a greater sensitivity of such tissues.

Photosynthetic activity is regarded as one of the most heat-sensitive plant metabolic process, and is often inhibited before other cell functions (Berry and Bjorkman 1980); photosystems (primarily PSII), the ATP generating system and carbon fixation pathways being amongst major stress-sensitive sites (Wahid et al. 2007; Allakhverdiev et al. 2008). If moderate heat stress causes a reversible reduction of photosynthesis, acute heat stress, even for only a short period, can lead to irreversible damages to the photosynthetic apparatus, resulting in the inhibition of plant growth. The impairment of photosynthesis can occur either through direct effects of the stress factor or via the inhibition of *de novo* protein biosynthesis by ROS accumulation (Allakhverdiev et al. 2008). It depends on the stage of growth of the photosynthetic tissue, with young developing and old senescing tissues exhibiting different thermo-tolerance (Kalituho et al. 2003; Zhang et al. 2012a; Marias et al. 2017a; Marias et al. 2017b).

Here, the exposure of *P. oceanica* plants to 34°C caused a significant rise of the minimal chlorophyll *a* fluorescence (F_0) in basal and medium leaf tissues that paralleled the depression of the ratio of variable fluorescence to maximum fluorescence (Fv/Fm), i.e. maximum photochemical efficiency of PSII. Differently from F_0, the decline of Fv/Fm under heat stress did not vary according to leaf height or rank (no significant interactions Heat×LH or LR were found). Basal fluorescence (F_0) and photochemical efficiency (Fv/Fm) are photo-physiological features commonly used as indicators of heat-induced thermal damages to PSII

in photosynthetic organisms (Yamada et al. 1996). Basal fluorescence, in particular, has been shown to correlate with photosynthetic thermo-tolerance in terrestrial higher plants (Knight and Ackerly 2002). In seagrasses, a recent study has shown that shallow and deep *P. oceanica* ecotypes exhibited different photo-physiological tolerance to heat stress (32°C), with deep ones evidencing lower tolerance. Accordingly, these plants displayed an augmented basal Chl *a* fluorescence (F_0), indicating they were experiencing critical temperature levels leading to photosynthetic injury and PSII inactivation (Marín-Guirao et al. 2016). Results presented here highlighted a differential heat-induced fluorescence along the *P. oceanica* leaf blade, which might indicate a differential thermo-tolerance of leaf-age segments.

At gene expression level, acute heat-stress caused a change in the expression of genes encoding components of the core complex of PSII: D1, D2 and CP43 (*psbA*, *psbD*, and *psbC*, respectively), although only for *psbA* results were significant. Interestingly, these changes varied with leaf rank; a significant suppression was observed only in the youngest leaf (i.e. rank 1), whereas leaves 2 and 3 exhibited values comparable to controls. This suggests a higher sensitivity of immature, developing leaves, and points for the inhibition of the PSII repair cycle in such tissues. The repair of PSII is a critical event that determines the tolerance of the photosynthetic apparatus to environmental stressors (Nishiyama and Murata 2014; Gururani et al. 2015). PSII activity is generally efficiently restored through the stimulation of the D1 protein degradation/removal and subsequent *de novo* biosynthesis (Ueno et al. 2016). However, when the rate of photodamage to PSII exceeds the rate of its repair, photoinhibition of PSII becomes apparent. In the current view, the suppression of the PSII repair cycle is attributed to the inhibitory action of ROS in the *de novo* protein synthesis and, in particular, of the D1 protein (Murata et al. 2007; Takahashi and Murata 2008; Nishiyama et al. 2011). The down-regulation of genes encoding for the two PSII core proteins D1 and D2 was also observed in deep *P. oceanica* plants submitted to heat stress by Marín-Guirao et al. (2016), whereas more tolerant shallow ecotypes were unaffected, as evidenced by unaltered *psbA* and *psbD* mRNA levels.

Contemporary to the significant rise of F_0 and subsequent decrease of Fv/Fm, an increase in non-photochemical quenching (NPQ) of chlorophyll fluorescence, was observed in heated plants, similar to what found in terrestrial plants (Yin et al. 2010). In particular, there was a tendency for basal, youngest leaf tissues of *P. oceanica*, to exhibit a lower capacity to dissipate excess excitation energy via NPQ, whereas middle and upper segments displayed higher NPQ values. At molecular level, NPQ induction was not supported by the up-regulation of *PSBS*. This gene was, indeed, highly significantly suppressed in heated

samples, and exhibited the lowest expression values in basal and intermediate segments of leaf 1. In recent years it has been demonstrated that a Psbs-independent induction of NPQ is still possible in *Arabidopsis* mutants lacking the PsbS protein, which was previously believed to be essential for this process (Johnson and Ruban 2011; Ikeuchi et al. 2014; Sylak-Glassman et al. 2014).

Acute heat-stress significantly slowed-down the photosynthetic electron flow (ETR), in all selected segments established along the *P. oceanica* leaf blade (a significant Heat×LH was found). When temperatures are slightly higher than optimal (moderate heat stress), the enhancement of electron transport and ATP synthesis, driven by increased activity of the Calvin cycle, can suppress the accumulation of excess electrons along the chloroplast electron transport chain, therefore lessening the production of ROS and enhancing PSII repair (Hancock Robert et al. 2013; Marín-Guirao et al. 2016; Ueno et al. 2016). On the contrary, reduced photosynthetic electron transport activity is observed in severely heat-stressed leaves (Wise R et al. 2004), accompanied by the down-regulation of transcripts and proteins associated with primary carbon assimilation, PSI, PSII, RuBisCO subunits, and electron transport proteins (Nouri et al. 2015).

Results presented here thus confirmed a severe impairment of the photosynthetic apparatus of *P. oceanica* at 34°C, with subsequent negative effect on plant growth. At molecular level, genes exhibiting the strongest suppression under intense warming were key components of the photosynthetic electron transport (*FD*), Calvin cycle (*RBCS*), light harvesting (*CAB-151*) and chlorophyll biosynthesis (*POR*). All of them were significantly affected by the factor heat, without any apparent interaction with the factor height and/or rank. However, youngest sections (basal and medium) of leaf 1 were undoubtedly the most negatively affected, as this pattern of down-regulation, although with a different extent, was persistent in such tissues. Conversely, leaves 2 and 3 showed a similar behavior and higher heat tolerance with respect to leaf 1, as evidenced by the slighter levels of down-expression of aforementioned genes. The strong repression of the gene encoding for the small subunit of the RuBisCO enzyme (*RBCS*) under heat stress, primarily in the youngest leaf tissues, and in a greater extent than PSII components, is of particular relevance. In fact, it has been well understood in recent years that PSII activity is not inhibited at temperatures that would inhibit whole leaf CO_2 assimilation, suggesting that CO_2 assimilation is more sensitive to heat stress (Salvucci and Crafts-Brandner 2004). Hence, this could be the reason behind the differential extent of gene down-regulation between core PSII components and the RuBisCO enzyme, particularly in leaf 1.

Photosynthetic pigment content of *P. oceanica* (Chl *a*, Chl *b* and carotenoids) was affected by the interaction Heat×LH. Both chlorophylls and carotenoids decreased significantly in *P. oceanica* at 34°C in uppermost leaf segments. However, also some leaf rank differences were detectable, with again leaf 1 exhibiting generally the lowest pigment concentrations, as for the H section of the leaf 3. Results obtained for *P. oceanica* are in agreement with many reports, indicating lesser accumulation of Chl in plants exposed to high-temperature stress, due to impaired Chl synthesis, its accelerated degradation or a combination of both (Kumar Tewari and Charan Tripathy 1998; Mathur et al. 2014). The impairment of Chl biosynthesis under heat stress results from down-regulation of gene expression and protein abundance of numerous enzymes involved in tetrapyrrole metabolism (Dutta et al. 2009), including Protochlorophyllide oxidoreductase, whose mRNA levels (*POR*) were strongly reduced also in *P. oceanica*.

One of the most important characteristic of thermo-tolerance is generally the massive production of HSPs (Qu et al. 2013). In *P. oceanica* at 34°C, a significant increase in transcript levels was observed only for *HSP90*, without a leaf height or rank interaction. On the contrary, *SHSP* showed a non-significant up-regulation. Notably, the most up-regulated genes of the overall gene expression dataset were *AOX* and *BI*, encoding for mitochondrial Alternative oxidase and Bax Inhibitor-1, respectively. They were significantly over-expressed in all leaf segments selected along *P. oceanica* leaves (significant Heat×LH interaction), and exhibited the highest induction in basal and medium sections, compared to upper leaf portions. The basal portion of the youngest leaf featured the highest fold changes among all considered leaf sections.

The induction of Aox and Bax inhibitor-1 under heat stress confirms their pivotal role in mediating seagrass stress acclimation, as well known in terrestrial plants; Aox pathway minimizes heat shock-mediated ROS production across the mitochondrial electron transport chain (Vanlerberghe 2013), while BI acts preventing ROS-induced programmed cell death (Watanabe and Lam 2006). Notably, Aox represents a link between metabolic activities and signaling, where it mediates the creation of a retrograde signaling network from the mitochondrion to the nucleus, which regulate stress-related gene expression (Saha et al. 2016). However, most of the genes involved in ROS-scavenging pathways, including AOX, showed higher expression levels during short-term heat shock, whereas their function in the long-term remains to be assessed (Qin et al. 2008).

Global DNA methylation analysis showed that % 5-mC increased in *P. oceanica* under heat stress, although such variation was only visible in intermediate (middle portion of leaf 2) and oldest leaf tissues (upper portion of leaf 3). Hence, DNA hypermethylation was detected

in leaf tissues showing higher thermo-tolerance at molecular and photo-physiological levels. Similarly, previous transcriptomic studies in *P. oceanica* revealed the exclusive activation of genes involved in epigenetic mechanisms (DNA and histone methylation) in more heat-tolerant shallow genotypes, with respect to less tolerant deep genotypes (Marín-Guirao et al. 2017). This confirms the involvement of the epigenetic machinery in the modulation of *P. oceanica* heat acclimation, as already demonstrated in terrestrial higher plants (Liu et al. 2015).

Epigenetic modifications, including DNA methylation, histone modifications, histone variants, ATP-dependent chromatin remodeling, histone chaperones, and small/long non-coding RNAs, can regulate the expression of heat-responsive genes and function to prevent heat-related damages (Liu et al. 2015). At whole genome level, DNA methylation has been shown to be differently affected by heat stress, in different species. For example, in *Arabidopsis*, heat exposure resulted in an increased global DNA methylation (Boyko et al. 2010), and similar results have been obtained for cork oak (Correia et al. 2013) and *Brassica* (Gao et al. 2014), resembling what has been found here for *P. oceanica*. However, it appears that there is no consistent trend in DNA methylation changes under heat stress in different species, since genome-wide hypomethylation has been also demonstrated (Min et al. 2014). Intriguingly, two previous studies in *P. oceanica* demonstrated whole genome hypermethylation in response to light-limitation stress and cadmium exposure (Greco et al. 2011; Greco et al. 2013), besides methylation changes at specific loci, as well as the up-regulation of a DNA chromomethylase, which is involved in both maintenance and *de novo* DNA methylation. Therefore, increasing whole DNA methylation level seems to be a recurring stress response in *P. oceanica*. Two main hypothesis on the significance of such DNA hypermethylation can be proposed: i) higher DNA methylation could suppress retrotransposition, which is triggered by environmental stressors (Mirouze et al. 2011); ii) increase in DNA methylation may down-regulate the expression of the transcriptome slowing down plant metabolism, which allows it to conserve energy needed to overcome the temporary challenge (Saraswat et al. 2017).

In conclusion, these data revealed, for the first time, the presence of differential age-dependent stress-induced epigenetic and gene-expression changes in *P. oceanica*, underlying photo-physiological and morphological responses to heat stress. Youngest leaf tissues exhibited lower thermo-tolerance, as evidenced by the dramatic down-regulation of key genes involved in photosynthetic electron transport, carbon assimilation and Chl biosynthesis, concurrently to the extreme over-expression of genes involved in alternative mitochondrial respiration and PCD suppression. Heat stress induced DNA methylation in

more tolerant leaf tissues, although the biological significance of this variation remains to be assessed.

Results presented in this thesis have clear methodological implications when assessing stress-induced effects on physiological and molecular properties in seagrasses. In fact, the "control analysis" confirmed that, in natural conditions, intermediate sections of leaves 2 and 3 are the most representative of the metabolic plant state, whereas short-term acute heat stress dramatically affected young, more than mature leaf tissues. Hence, physiological and molecular evaluations conducted only on such tissues, as common practice, would give unreliable estimates of the actual plant state.

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Appendix II

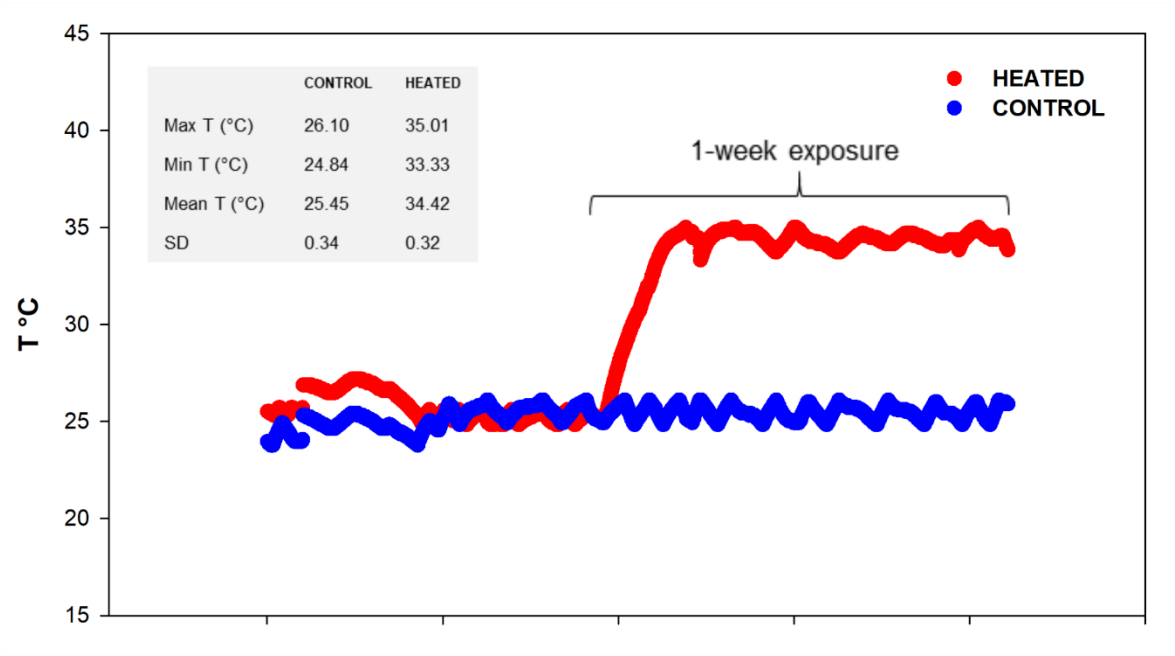

Fig. A2.1 Scatter plot of temperature (°C) values measured all along the experiment. Blue and red dots represent T values in control and heated tanks, respectively. Max, min and mean T values are indicated in the grey box.

Table A2.1 Photosynthetic parameters and pigment concentrations determined in B, M and H sections of leaves 1, 2 and 3, under control (C) and heated (H) conditions. R-ETR (µmol electrons m^{-2} s^{-1}); Chl *a*, *b* and carotenoids (µg cm^{-1}); Chl *b/a* (molar ratio). Values are means (SE) for *n*=3.

	F_0		Fv/Fm		r-ETR		NPQ		Chl *a*		Chl *b*		Carotenoids		Chl *b/a*	
	C	H	C	H	C	H	C	H	C	H	C	H	C	H	C	H
Leaf 1																
B	332.33 (12.50)	347.17 (19.44)	0.77 (0.00)	0.65 (0.02)	15.77 (2.07)	9.87 (0.99)	0.50 (0.16)	1.00 (0.08)	21.48 (3.52)	16.25 (0.91)	10.62 (1.64)	7.58 (0.40)	5.97 (0.84)	4.28 (0.18)	0.49 (0.02)	0.46 (0.00)
M	343.50 (3.97)	411.50 (26.70)	0.77 (0.00)	0.61 (0.03)	23.47 (2.21)	12.88 (0.90)	0.69 (0.05)	1.58 (0.18)	30.75 (1.89)	25.90 (2.98)	14.86 (1.18)	13.24 (1.28)	8.44 (0.65)	6.73 (1.04)	0.48 (0.01)	0.51 (0.06)
H	380.67 (10.89)	373.17 (40.42)	0.74 (0.01)	0.52 (0.03)	23.12 (1.36)	11.80 (0.85)	1.39 (0.21)	3.46 (0.53)	34.27 (2.34)	23.00 (2.86)	17.01 (1.26)	11.07 (1.12)	9.99 (0.72)	7.18 (0.43)	0.49 (0.01)	0.48 (0.03)
Leaf 2																
B	372.50 (8.54)	403.17 (17.45)	0.77 (0.00)	0.68 (0.04)	16.20 (1.66)	11.58 (2.05)	0.51 (0.08)	0.83 (0.06)	20.04 (0.81)	19.88 (1.08)	10.78 (0.47)	9.34 (0.59)	4.91 (0.40)	5.57 (0.73)	0.51 (0.04)	0.46 (0.01)
M	365.50 (2.75)	471.33 (6.98)	0.77 (0.00)	0.63 (0.03)	23.03 (0.48)	12.90 (1.60)	0.64 (0.04)	1.38 (0.23)	29.84 (2.40)	28.43 (3.94)	15.11 (1.14)	13.19 (1.73)	8.94 (0.98)	7.94 (0.95)	0.50 (0.02)	0.46 (0.01)
H	372.00 (4.44)	412.40 (9.22)	0.75 (0.00)	0.53 (0.02)	24.25 (0.84)	11.33 (1.26)	0.94 (0.13)	3.82 (0.54)	33.80 (2.46)	27.18 (3.68)	17.01 (1.27)	13.40 (1.76)	10.13 (0.67)	8.24 (0.47)	0.50 (0.01)	0.49 (0.00)
Leaf 3																
B	389.83 (8.09)	512.67 (42.59)	0.76 (0.00)	0.61 (0.05)	16.82 (2.20)	9.87 (1.27)	0.60 (0.08)	0.94 (0.26)	21.17 (1.21)	19.88 (0.16)	10.47 (0.58)	10.55 (0.60)	5.35 (0.37)	4.75 (0.51)	0.49 (0.00)	0.51 (0.02)
M	423.50 (10.69)	482.00 (15.45)	0.74 (0.01)	0.60 (0.01)	20.00 (0.81)	12.97 (1.08)	0.85 (0.05)	2.67 (0.34)	28.45 (3.08)	26.52 (1.43)	14.26 (1.70)	14.28 (1.27)	7.64 (0.85)	6.58 (0.17)	0.49 (0.01)	0.53 (0.03)
H	419.50 (32.00)	413.00 (13.80)	0.71 (0.03)	0.55 (0.05)	22.09 (1.83)	9.73 (3.14)	1.78 (0.32)	4.65 (1.15)	34.44 (1.32)	16.84 (0.72)	17.39 (0.46)	8.73 (0.21)	10.56 (0.48)	6.40 (0.94)	0.50 (0.01)	0.51 (0.01)

Chapter III - Organ and shoot type-specific variations in response to light limitation in *P. oceanica*

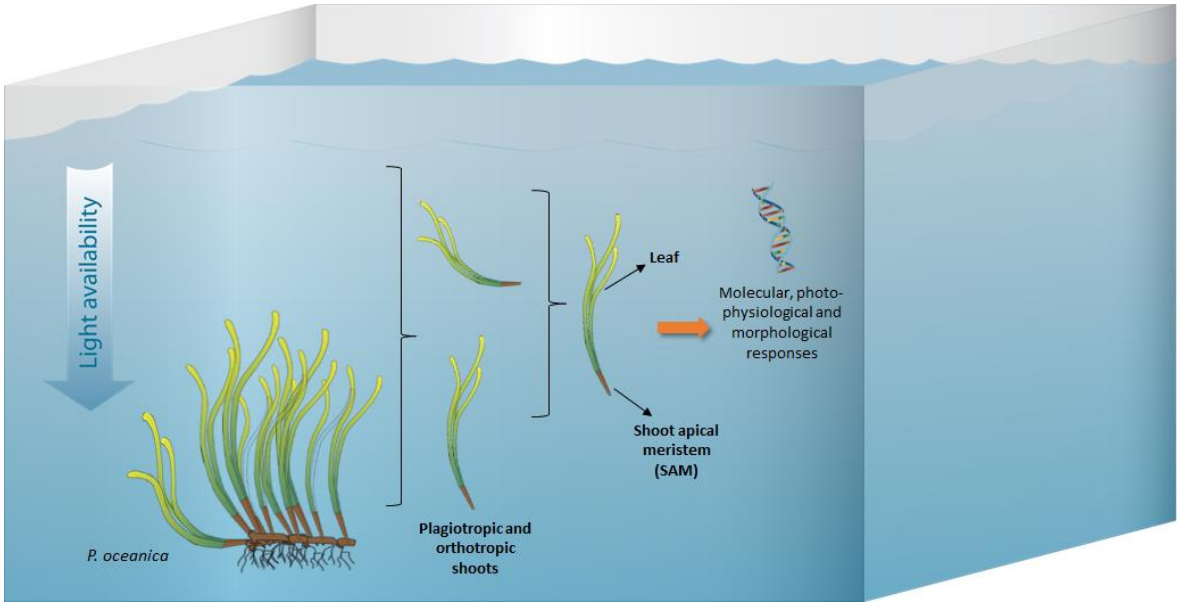

Fig. 3.1 Conceptual diagram of the experiment presented in this chapter. Organ and shoot type-specific variations in response to light limitation in *P. oceanica*. (All symbols taken from http://ian.umces.edu/imagelibrary/).

3.1 Introduction

3.1.1 *Clonal integration*

All plants are modular organisms. When these modules are capable of iterating themselves in an independent manner, thus producing offspring through vegetative propagation, the plant is referred to as clonal (Liu et al. 2016). As already detailed in the Chapter I, these clonally formed offspring are called 'ramets'(Harper 1977), whereas the whole plant, which can be comprised of a number of ramets, is referred to as a 'genet' (Harper 1977). Different ramets belonging to the same genet share the same genotype (Harper 1977). Within a genet, each ramet has the potential to perform all biological functions, and can be regarded as an independent individual.

Clonal plants dominate diverse terrestrial and marine ecosystems as primary producers, comprising many of the most important crops and invasive plants, and some of earth's largest, tallest, and oldest plant species (Douhovnikoff and Dodd 2015). Clonal integration, i.e., the physiological integration taking place among the different ramets for sharing resources and information, is a striking attribute of clonal plants, which plays a role in their ecological and evolutionary success, and enables them to act as a cooperative system (Liu et al. 2016). This is possible since ramets are physically linked each other through horizontal structures (e.g. rhizomes or stolons) allowing the translocation of various material, including external resources absorbed by plants (e.g. water and nutrients), hormones, photosynthates, and secondary metabolites, via interconnected vascular structures (Liu et al. 2016). Clonal integration permits plants to cope with spatiotemporal heterogeneity of the environment. For example, within a single genet, donor ramets situated in favorable microsites (e.g. with abundant resource supply) can help resource-poor or otherwise adversely placed ramets, to alleviate their shortages (e.g. shading, nutrient depletion and drought) and/or to tolerate abiotic and biotic stressors (Liu et al. 2016). This has been often observed from parent ramets (older) to offspring ramets (younger/developing), however reciprocal exchange of resources between neighboring ramets growing in differing-quality patches has also been described (Alpert 1999). Ultimately, resource sharing through clonal integration results in an increased performance of the recipient part without decreasing that of donor parts (at least in the short-term), thus leading to an increased performance of the whole clone (Song et al. 2013). Numerous studies have showed that clonal integration can support ramets to survive in stressful environments, for instance under high salinity (Evans and Whitney 1992; Pennings

and Callaway 2000), soil alkalinity stress (Zhang et al. 2015) or to withstand defoliation by herbivores (Schmid et al. 1988; Wang et al. 2017).

As outlined in Chapter I, seagrasses are clonal rhizomatous plants sharing a similar morphology to that of terrestrial monocotyledons. All seagrass species present a highly organized growth, which relies on the reiteration of ramets, which are composed of modules: a bundle of leaves, a piece of rhizome, and a root system. Rhizomes are stems extending either horizontally on (or below) the sediment surface or vertically, raising the leaves towards, or above, the sediment surface (Marbà et al. 2004). Besides providing mechanical support and nutrient storage, rhizomes are responsible for the extension of the seagrass clone in the space, as well as for connecting adjacent ramets, thus enabling physiological integration (Marbà et al. 2004). *P.* oceanica has dimorphic rhizomes; hence, it possesses both horizontal (plagiotropic) rhizomes, and vertical (orthotropic) rhizomes, whereas other species such as *Zostera* spp. have only horizontal rhizomes. Shoots that are born by rhizomes growing vertically and horizontally are called orthotropic and plagiotropic shoots ("runners" or apical shoots), respectively. Seagrass beds typically have wide spacing between many vertical shoots with few horizontal apices, and are able to spread through those apices (i.e. apical dominance) (Terrados et al. 1997a), which grow horizontally until space has been completely colonized. Plagiotropic shoots can revert into vertical, which leads to the cessation of their horizontal growth, or vertical shoots can branch to produce horizontal ones, when the apical meristem of the original horizontal rhizome dies (Marbà et al. 2004). Clonal integration has been demonstrated in seagrasses, for example in the form of nitrogen and carbon translocation among neighboring ramets (Marbà et al. 2002). Photosynthates and nutrients are known to be re-allocated within seagrasses mainly toward organs with high metabolic activity, including growing leaves, flowering shoots and remarkably apical shoots, thus resulting in enhanced clone growth and meadow spreading (Harrison 1978; Libes and Boudouresque 1987; Terrados et al. 1997b; Marbà et al. 2002; Marbà et al. 2006; Schwarzschild and Zieman 2008a; Schwarzschild and Zieman 2008b). Clonal integration supports seagrass persistence, ameliorating adverse effects of environmental stressors. For example, Tuya et al. (2013a,b) demonstrated that the preservation of clonal integration in *C. nodosa* buffered its physiological performance against small-scale burial events and nutrient enrichment, similar to what observed for *T. testudinum* under localized light limitation (Tomasko and Dawes 1989). The importance of clonal traits was also revealed in *Z. noltii* grown under low light conditions and organic matter enrichment (Olivé et al. 2009). Specifically, a differential plant response was observed when contrasting levels of organic matter and light were established between plant apex and distal part, with harmful effect of

organic matter being alleviated when the apex was grown in high light. This demonstrated that apical shoots are the leading plant parts, and are more sensitive to light deprivation (Olivé et al. 2009).

3.1.2 *Shoot-apical meristem*

Plants, in contrast to animals, grow and continuously generate new organs and tissues after embryogenesis, an ability that helps them to deal with environmental changes. This flexibility in development and organogenesis is possible thanks to the activity of specialized structures called meristems that contain pools of stem cells, and are maintained throughout their lifespan (Carraro et al. 2006; Brukhin and Morozova 2011). Two main meristems exist in plants, namely the shoot-apical meristem (SAM), responsible of generating all above-ground tissues and organs (e.g. stems and leaves), and the root-apical meristem (RAM), which give rise to all below-ground parts (e.g. root system) (Carraro et al. 2006). The RAM and the SAM display different structural organizations, but both harbor stem cells that are maintained in a pluripotent state by signals from the neighboring cells (Brukhin and Morozova 2011).

In the SAM, as the stem cells divide, some daughter cells are displaced toward the periphery to produce lateral organs, while others are retained at the shoot apex to replenish the stem cell reservoir (Carles and Fletcher 2003). Whatever the developmental stage, the meristem must keep this delicate balance between self-renewal of stem cells and continuous organ initiation by peripheral cells (Carles and Fletcher 2003). Both organogenesis activity and SAM maintenance are dynamically controlled by complex, multifactor and overlapping signaling networks that include the feedback regulation of meristem maintenance genes (e.g. the CLAVATA pathway) as well as informative cues from plant hormones (e.g. cytokinins, gibberellins and auxins) (Murray et al. 2012). Many of the genes involved in SAM functions are widely conserved among plant species (Bäurle and Laux 2003; Carles and Fletcher 2003), although most information are available for a restrict number of terrestrial model plants, primarily *A. thaliana*, but also monocots like rice and maize.

As fundamental plant structures ensuring organogenesis over the whole plant's life, meristems are particularly sensitive to environmental hazards such as drought, high salinity or heavy metals that can cause oxidative stress, and consequently, DNA damage and mutations in these crucial cell populations (Fulcher and Sablowski 2009). Particularly in the shoot apical meristem, somatic mutations within the stem cell pool, can become fixed and contribute to the germline, thus affecting reproductive fitness (Fulcher and Sablowski 2009).

Therefore, plants evolved special mechanisms to protect these cell niches from DNA damage and safeguard genome integrity, including cell-cycle arrest, DNA repair and ultimately selective PCD programs to eliminate damaged cells from the population of stem cells and their early descendants, that are different from those of differentiated cells (Hefner et al. 2006; Fulcher and Sablowski 2009).

3.1.3 *The study*

The study presented here aims at disentangling the effects of acute low-light stress on *P. oceanica*, considering the response of: I) plagiotropic *vs.* orthotropic shoots and II) leaf *vs.* meristem tissues.

Light availability is by far the most important factor controlling seagrass growth, survival, and depth distribution (Lee et al. 2007; Ralph et al. 2007). This is attributed to the fact that the minimum light requirement for seagrasses is one of the highest among all angiosperms (see Chapter I for more details), therefore any further attenuation due to natural and/or anthropogenically-driven processes can compromise the photosynthetic process and ultimately lead to seagrass loss, as already documented worldwide (Short and Wyllie-Echeverria 1996; Ralph et al. 2006). Underwater irradiance attenuation occurs naturally along several gradients, namely the bathymetric, the canopy, and the leaf-epiphytic gradients. In addition, light attenuation may occur indirectly through excess anthropogenic nutrients leading to eutrophication, increased sediment accretion and resuspension, aquaculture and dredging, as well as regional weather patterns (e.g. extreme storms and altered rainfall events) (Ralph et al. 2007).

Seagrass responses to light limitation at multiple level of organization, from molecular to physiological and morphological levels, and across various spatial scales, from leaf to meadow scale, has been deeply addressed, and plenty of information are available for several species (Ralph et al. 2007; Davey et al. 2016; Kumar et al. 2016; Malandrakis et al. 2017; Davey et al. 2018) (see also Chapter I). Nonetheless, physiological studies conducted so far investigated the effect of low-light conditions only on seagrass leaf tissues, whereas the effect on SAM has been disregarded, although the function of this meristematic structure is fundamental for seagrass to ensure growth and survival under abiotic and biotic stressors. Yet, the analysis of SAM-related gene expression could be much more informative of arising cellular stress than leaf-related gene expression, since these cells have a consistent low threshold for activation of repairing processes (e.g. induced DNA repair mechanisms), and a general hypersensitivity to DNA damage (Fulcher and Sablowski 2009).

Another aim of the work was to understand the differential response to light limitation of apical (or plagiotropic) shoots, that are considered the leading plant parts, responsible for colonization and clone extension, in respect to vertical (or orthotropic) shoots, that possess a different biological role, as discussed in 3.1.1. In particular, I wanted to investigate the hypothesis that molecular signals of clonal integration would be seen when the transcriptome profile of these two types of shoot is compared under stress conditions.

To address these questions, the seagrass *P. oceanica* was exposed to a medium-term acute low-light (LL) stress for 40 days in a mesocosms system, whereas control samples were maintained to light levels resembling environmental conditions experiences by the natural population during the study period. Whole transcriptome analysis was performed via Illumina RNA-Seq on leaves and SAMs of both plagiotropic and orthotropic shoots, in control and LL conditions. Molecular analyses paralleled photo-physiological (photosynthetic parameters and pigment content) and morphological assessments. Fitness-related traits (leaf growth rate and necrosis marks) and shoot mortality, were also determined under control and LL.

3.2 Materials and Methods

Experimental design

For this study, large *P. oceanica* fragments bearing several orthotropic shoots and at least one plagiotropic shoot, were collected by SCUBA diving from a shallow-water meadow (8–10 m depth) located around the island of Ischia (Gulf of Naples, Italy 40°43.849' N, 13° 57.089' E) on 16th February 2018 (11:00-12:00 pm). Plant material was kept in darkened coolers filled with ambient seawater and rapidly transported to the laboratory (within 1-2 hr) to be immediately transplanted in the indoor mesocosm facility of Stazione Zoologica Anton Dohrn (Naples, Italy) described in Chapter II. Twenty-four plant fragments of similar size and shoot number (15-25 connected shoots) were selected to standardise the experiment, and individually attached to the bottom of twelve plastic net cages (40x30x10 cm) filled with coarse sediment (two fragments per pot). Two randomly selected cages were then placed in each of the six glass aquaria (500L) (see Chapter II). As for the previous experiment, large fragments of *P. oceanica* were preferred over small ones to ensure healthy conditions of plants during the experimental period (Marín-Guirao et al. 2011) and to resemble the canopy structure of the meadow. Details about the mesocosm system and water quality monitoring methods can be retrieved from Chapter II (2.2).

Prior to start the experimental treatment, plants were acclimated for 10 days to the mean prevaling environmental conditions of the sampling site during the study period (temperature: ca. 16.5 °C; salinity: 37.5 psu; max. noon subsurface irradiance: ca. 200 µmol photons m^{-2} s^{-1}; 11 h:13 h light:dark photoperiod). Subsequently, irradiance level in half of the tanks was lowered to 40 µmol photons m^{-2} s^{-1} resambling a strong shading event (low-light stress), whereas lamps of control tanks were maintained at ca. 210 µmol photons m^{-2} s^{-1} (Fig. 3.2). Both values represent max. noon irradiance levels. Temperature and salinity levels were left as in the acclimation phase (T: ca. 16.5 °C; salinity: 37.3-37.7 psu). Continuous light and temperature measurements were performed all along the experiment by means of sensors described in Chapter II (2.2), salinity was kept within the range indicated above by regular additions of freshwater.

The low-light exposure lasted 40 days. Chlorophyll *a* fluorescence-derived photosynthetic parameters and pigment content were determined after 15 days (T1) and 30 days (T2) of exposure on both plagiotropic and orthotropic shoots (Fig. 3.3).

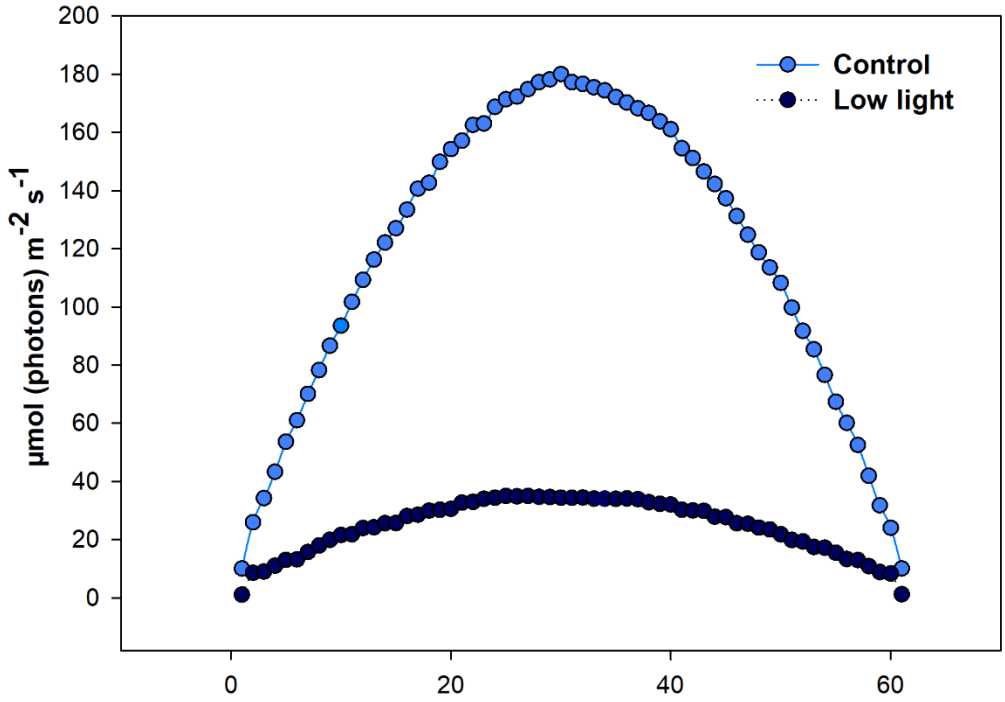

Fig. 3.2 Daily irradiance at the top of the leaf canopy measured with LI-COR LI-1400, in control (light blue) and low-light (dark blue) tanks, scaled over a one-hour measurement. Y-axis represents irradiance level in µmol photons m^{-2} s^{-1}; X-axis represents time (minutes).

Plant morphology, fitness-related traits and shoot mortality were assessed at T1, T2, and at the end of the experimental period (T3, 40 days of exposure) (Fig. 3.3). Genome-wide transcriptome analysis (RNA-Seq) of control and low-light exposed plants, was exclusively performed at T1 on leaves and rhizome tips (containing the SAM) of both plagiotropic and orthotropic shoots (Fig. 3.3). RNA-seq analysis was performed only at T1 since one of the aim of this study was to identify early signal of plant stress, anticipating morphological changes and ultimately shoot mortality events. Photo-physiological measurements and pigment content were always determined on middle section of mature *P. oceanica* leaves (rank 3) of plagiotropic and orthotropic shoots. Pictures of the mesocosm system, including control and low-light tanks, and examples of plagiotrophic and orthotropic shoots and rhizome tip employed for this experiment are shown in Fig. 3.4 (A-E), Fig. 3.5 (A-D) and Fig. 3.6.

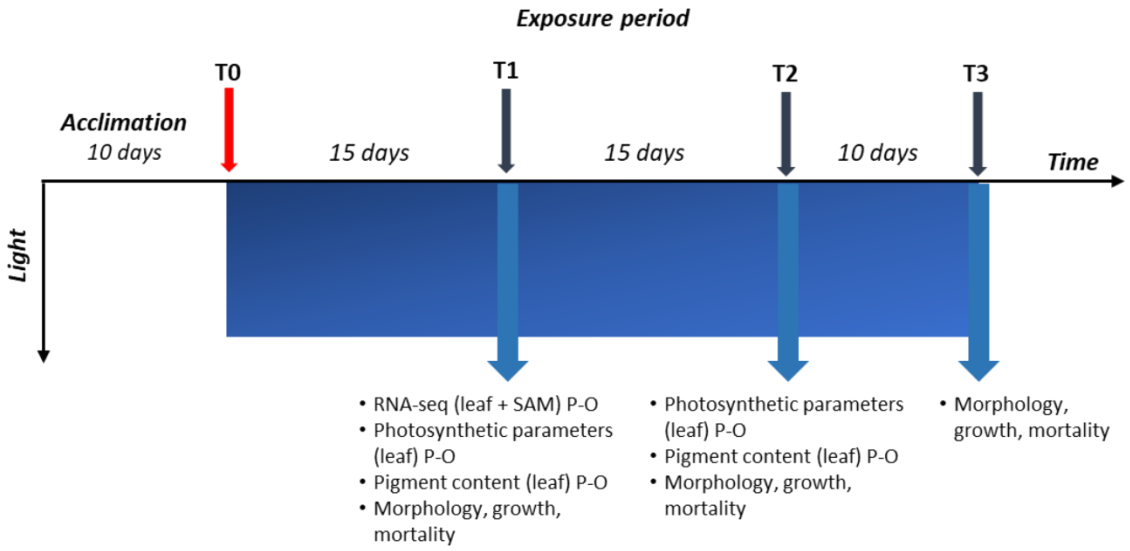

Fig. 3.3 Graphical depiction of the experimental design. Acclimation and sampling time points during the exposure phase are showed. All analyses performed at each time point are indicated below the blue arrows. Red arrow represents the start of the low-light treatment. "P-O" stands for plagiotropic and orthotropic shoots.

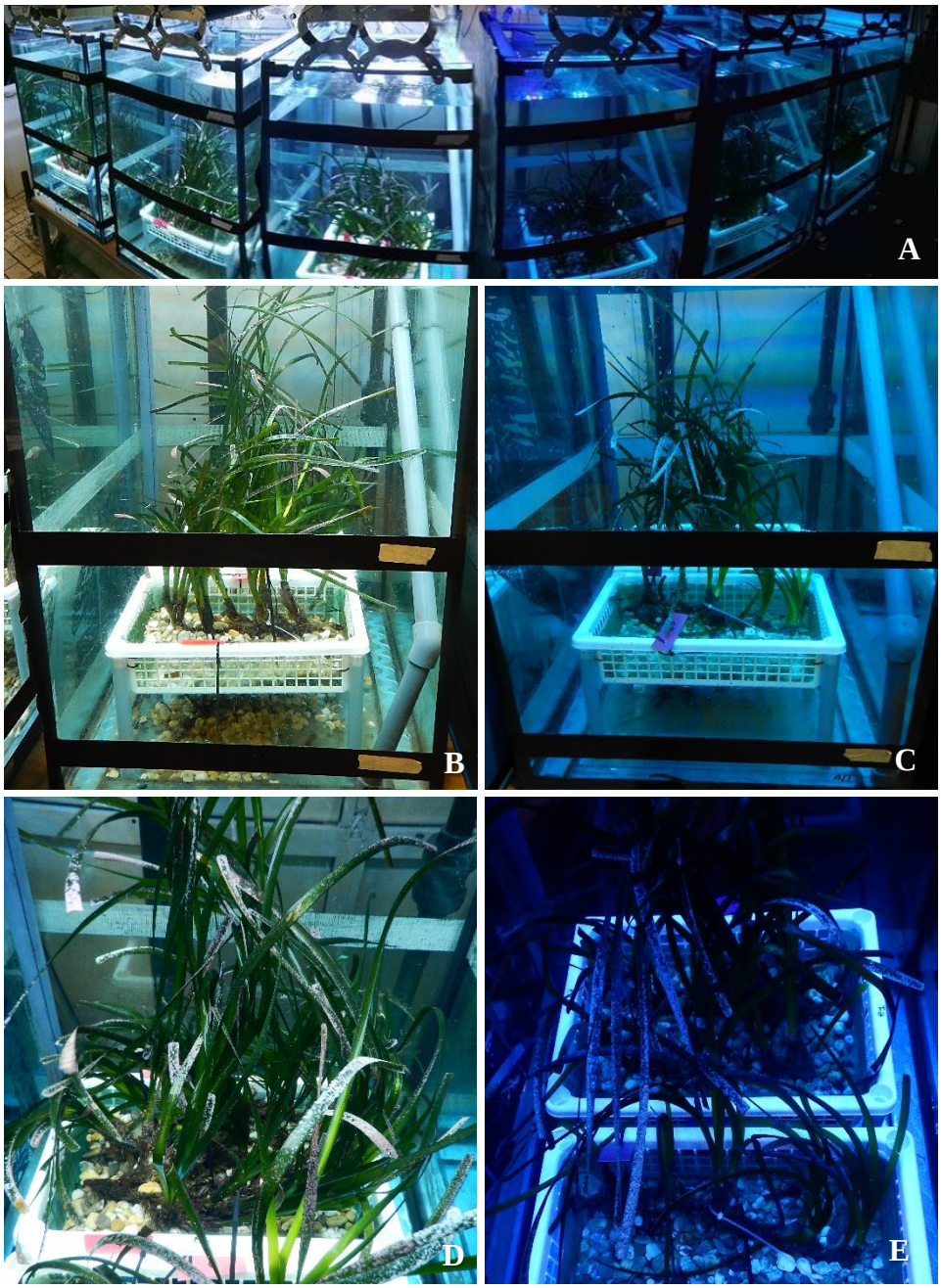

Fig. 3.4 (A) Panoramic view of the experimental system at SZN; In (B) and (C) an example of a control and low-light tank, respectively; (D) and (F) underwater photos depicting *P. oceanica* fragments placed in control and low-light tanks. Photo credit: M. Ruocco and G. Procaccini.

Fig. 3.5 (A) Example of two plagiotropic shoots (indicate with arrows) and (B) three orthotropic shoots used for this experiment; In (C) and (D) open view of one of the above plagiotropic and orthotropic shoots, respectively. Photo credit: M. Ruocco

Fig. 3.6 Example of a rhizome tip containing the SAM (indicate with arrow) and open view of a vertical *P. oceanica* shoot used for this experiment. Photo credit: M. Ruocco.

Shoot morphology, growth and survival

A set of vegetative variables (shoot size, number of leaves per shoot, maximum leaf length and width, necrotic leaf surface, leaf growth rate and net shoot change) were determined at T1, T2 and at the end of the experiment (T3) to estimate morphology of plagiotropic and orthotropic shoots, fitness traits and plant survival under low-light conditions. To determine leaf growth rate, all apical and vertical shoots of one rhizome fragment per tank (at least one apical and three vertical shoots) were marked at the beginning of each experimental phase (T0, T1 and T2) following the Zieman method described in Chapter II (2.2). Marked fragments were then harvested at the end of each experimental phase (T1, T2 and T3) to determine mean values of leaf growth (i.e. newly formed tissue below the needle mark; cm^2 of new tissue $shoot^{-1}$ day^{-1}) for plagiotropic and orthotropic shoots, separately. All other morphological features were measured on the same shoots and time points. To determine plant mortality, all shoots (without distinguish apical and vertical ones) in each tank were

counted at the beginning and at the end of the experimental phases, and the differences were normalized to initial shoot numbers and expressed as a percentage of net change. Negative values indicated a net decline from the initial shoot number. Within each tank, obtained values were averaged and used as individual replicates (*n*=3).

Photo-physiology and pigment content

Chlorophyll *a* fluorescence measurements were performed with a diving-PAM fluorometer (Walz, Germany) as described in Chapter II (2.2). The saturation pulse method was used to measure F_0, Fm and to calculate the maximum photochemical efficiency of PSII (Fv/Fm) in plagiotropic and orthotropic shoots of plants adapted to the dark throughout the night. RLC method was subsequently applied on the same ramets and leaf area after 5 hours under illumination in the aquaria. The effective quantum yield of PSII (ΔF/Fm'), relative electron transport rate (r-ETR), minimum saturating irradiance (Ik) and non-photochemical quenching (NPQ), were obtained from RLCs (see Chapter II (2.2) for further details).
About 5 cm-tissue sections from the middle portion of mature leaves (rank leaves 3) were collected from plagiotropic and orthotropic shoots and used for pigment analyses. Pigment extraction and determination of chlorophyll *a*, *b* and total carotenoid concentration was carried out as described in Chapter II (2.2) and expressed as μg cm^{-2}. Non-invasive chlorophyll *a*-derived photosynthetic measurements were determined on two plagiotropic and two orthotropic shoots per tank, then values were averaged to be used as individual replicates. This means that the number of replicates used in statistical tests was *n*=3 (total biological replicates N=6). Pigment concentration was instead determined on one orthotropic and plagiotropic shoot per tank (*n*=3).

Genome-wide transcriptome sequencing and analysis

RNA extraction, library preparation and sequencing: Leaf sub-samples (ca. 5 cm) for RNA extraction were obtained from middle section of mature leaves (rank 3) of the same orthotropic and plagiotropic shoots employed for pigment content analysis (*n*=3). In addition, after the excision of the shoot, the first most apical 0.5 cm of the rhizome tip, containing the SAM, were also collected from the same P-O shoots (*n*=3). Leaf material was gently cleaned from epiphytes and submerged in RNAlater© tissue collection (Ambion, life technologies), then stored as outlined in Chapter II (2.2). Rhizome fragments were cleaned from leaf sheaths and sediment particles and then preserved in LN2 to be definitely stored at -80 °C until RNA extraction. Total RNA was extracted as outlined in Chapter II (2.2). The

purity of the total RNA was checked using NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific) and 1.0% agarose gel electrophoresis. RNA was used only when Abs260 nm/Abs280 nm and Abs260 nm/Abs230 nm ratios were >1.8 and 1.8<×<2, respectively. RNA concentration was accurately determined by Qubit® RNA BR assay kit using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific). RNA quality was calculated by measuring the RNA Integrity Number (RIN) with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.); only high-quality (RIN ≥ 7) RNA was used for RNA-Seq analysis. Quality checked RNA samples from *P. oceanica* leaves and SAMs were sent to Genomix4life s.r.l. (Salerno, Italy) for libraries' preparation and sequencing. Twenty-four indexed cDNA libraries (2 shoot types × 2 organs × 2 treatments × 3 biological replicates) were constructed with the Illumina TruSeq® Stranded mRNA Library Prep Kit, and sequenced with an Illumina NextSeq 500 platform (single-ends 1×75 cycles; ~20,000,000 total reads/sample).

RNA-seq data quality-check, assembly and differential expression analysis have been performed by Dr. Laura de Entrambasaguas Monsell.

Data filtering and transcriptome assembly: Raw sequencing data were checked using FastQC (v0.11.5) software (Andrews 2010), and then cleaned for Illumina adaptors and trimmed for quality using Trimmomatic (v0.36) (Bolger et al. 2014). Only reads with a minimum length of 50 bp were retained. After raw data were adapter- and quality-trimmed and filtered, an average of 20,750,713 high-quality reads for each library were obtained, for a total of 421,487,471 reads (84.63% of row reads) (Table A3.1 in Appendix III). Subsequent transcriptome assembly was conducted using the Trinity pipeline (v.2.5.0) (Haas et al. 2013) with default parameters. Intra-assembly redundancy was decreased by using CD-hit-EST v4.6.7 (Huang et al. 2010). This newly assembled transcriptome was combined with three previously published *P. oceanica* transcriptomes (D'Esposito et al. 2017; Entrambasaguas et al. 2017; Marín-Guirao et al. 2017) into one merged assembly, and highly similar contigs were clustered by similarity using CD-hit-EST algorithm. To further evaluate the quality of the assembled transcriptome (1) assembly statistics using the TrinityStats.pl from the Trinity package, (2) the proportion of reads mapping back to the transcriptome assembly using Bowtie v1.1.1 (Langmead et al. 2009), and (3) the number of contigs longer than 1Kb, were computed.

Functional annotation, differential expression and GO enrichment analysis: Assembled contigs were annotated through sequence similarity search against UniProtKB/Swiss-Prot and NCBI non-redundant sequence (Nr) protein databases using BLASTX program implemented in BLAST+ tool v2.6.0 (Altschul et al. 1997) (e-value cutoff 1e-6).

Subsequently, results were loaded on Blast2GO v.5 (Conesa et al. 2005) to retrieve Gene Ontology (GO) terms (e-value cutoff 1e-6) for transcripts with a positive BLAST hit. Enzyme code (EC) annotation and KEGG maps for the metabolic pathways in which they are involved were also retrieved. For the differential gene-expression analysis, reads from each biological replicate were individually mapped to the assembled transcriptome using the Bowtie v1.1.1 aligner (Langmead et al. 2009), and expression of each transcript was quantified using the Expectation-Maximization method (RSEM) (Li and Dewey 2011). Finally, differentially expressed genes (DEGs) for each pairwise comparison were determined using a Generalized Linear Model (GLM) in the edgeR package (Robinson et al. 2010). In order to remove the bulk of low-abundance genes, very lowly expressed genes were removed keeping those having at least a cpm (read/count per million) of 1 or greater for at least three samples (the size of the smallest group of replicates). Transcripts were considered significantly differentially expressed (up- and down-regulated) if FDR-corrected *P* value < 0.05 and -2 < FC < +2. When multiple isoforms were present for a given gene, the longest was defined as the gene functional annotation. Expression values generated by edgeR were used for examining profiles of expression across different samples through a hierarchical clustering. A heatmap of DEGs was generated using the heatmap3 package in R v3.2.2 (R Core Team 2015). To assess overall similarity across samples and discard possible discrepancies, sample relationships were explored through a PCA on the transposed normalized expression matrix with R v3.2.2. Venn diagrams to identify shared and unique DEGs between different contrasts were performed with http://bioinformatics.psb.ugent.be /webtools/Venn/.

Gene Ontology (GO)-term enrichment analysis of DEGs of each pairwise comparison was performed through the Fisher's exact test approach by using the GO enrichment analysis function provided by Blast2GO v.5, with a threshold FDR of 0.05. The analyses were carried out by comparing the GO terms of DEGs of all comparisons with the GO terms in a background reference (newly assembled transcriptome). Due to a large list of enriched GO terms was obtained in most comparisons, a further reduction to most specific terms was carried out. Summarization and visualization of GO terms were performed by using the REVIGO web service (http://revigo.irb.hr/) (Supek et al. 2011).

Data analysis

Multivariate statistics was used to assess the overall signal of all photo-physiological variables (photosynthetic parameters and pigment content). Specifically, a PERMANOVA was conducted with the Primer 6 v.6.1.12 & PERMANOVA + v.1.0.2 software package (PRIMER-E Ltd) (Clarke and Gorley 2006). The analysis was run for each time point (T1 and T2) separately, and consisted of two fixed factors: "Shoot Type" (ST), with two levels (plagiotropic – P and orthotropic – O) and "Light" (L), with two levels (control – C, low light – LL). A PCA was also performed for the multivariate photo-physiological dataset with the software PAST v.3.03 (Hammer et al. 2001). Two-way ANOVAs were conducted to detect the effects of shoot type and treatment on single photo-physiological variables (chlorophyll *a* fluorescence-derived photosynthetic parameters and pigment content) and vegetative traits (shoot morphology and leaf growth rate) with the same aforementioned levels. Net shoot change was analyzed with one-way ANOVA, considering only "Light" as fixed factor. Normality of data was tested using the Shapiro-Wilk test and variance homogeneity was verified using Levene's test. When parametric assumptions were not met, data were Box-Cox transformed. Student-Newman-Keuls post-hoc tests was used whenever significant differences were detected. All ANOVAs were performed using the statistical package STATISTICA (StatSoft, Inc. v. 10).

3.3 Results

3.3.1 *Morphological and photo-physiological variations of plagiotropic and orthotropic P. oceanica shoots under low light*

Plant morphology, growth and mortality

A two-way ANOVA was used to reveal the effect of shoot type and light on plant morphological characteristics and leaf growth rate. Individual factors had varying effects on analyzed vegetative variables, without a significant interaction. As expected, plagiotropic shoots generally contained a significantly higher number of leaves per shoot and a lower maximum leaf length, in respect to orthotropic ones (Tables 3.1 and 3.2; Fig. 3.5 A-D). Both variables decreased in P-O shoots under LL exposure, although such variations were not significant at any sampling time points (Table 3.1 and 3.2). LL had mild effect on maximum leaf width, with a significant reduction observed at T1 (ca. 5%) and T3 (ca. 6-7%), with no significant differences between apical and vertical shoots (Table 3.1 and 3.2). LL exposure caused a global reduction in the shoot size that was especially evident after 30 and 40 days of exposure (T2 and T3), for both plagiotropic (ca. 31%) and orthotropic (ca. 26-27%) shoots (Table 3.1 and 3.2).

Leaf necrotic surface was not significantly affected by the experimental treatment (Table 3.2), although there was a tendency for LL plants to increase total necrotized tissue at T2 and T3 (Table 3.1). LL greatly slowed down leaf growth rate, as it was significantly reduced at all sampling time points (T1, T2 and T3), in shaded with respect to control plants (Table 3.2). More specifically, already after 15 days of exposure to 40 µmol photons m^{-2} s^{-1}, leaf growth was reduced by 50% and 41% in apical and vertical shoots, respectively (Table 3.1). After one-month exposure, a further decline of up to 62% and 55% in apical and vertical shoots, was observed. At the end of the experiment (T3, 40 days of exposure) leaf growth rate of apical shoot was 78% lower than controls, whereas for vertical shoots the decrease was still around 50%, similarly to what observed at T2 (Table 3.1). It must be noted that, although there was no significant interaction ST×L, the reduction in leaf growth rate was always greater in plagiotropic than orthotropic shoots (Table 3.1). Control and LL plants showed a slight shoot decline (but not significant) along the experiment (Table 3.1). Shoot mortality progressively increased in LL exposed plants, from ca. 1% to 3% and 5% at T1, T2 and T3, respectively (Table 3.1), whereas control plants showed about 1% or no net changes in the number of shoots at the selected sampling time points (Table 3.1).

Table 3.1 Plant morphological characteristics (shoot size, number of leaves per shoot, maximum leaf length and width, necrotic leaf surface) and leaf growth in plagiotropic and orthotropic shoots, and total net shoot change at T1, T2 and the end of the exposure period (T3). Values are means (SE) for *n*=3. Results of ANOVA analyses for each sampling time are reported in Table 3.2. P_C = plagiotropic shoots, control; O_C = orthotropic shoots, control; P_LL = plagiotropic shoots, low light; O_LL = orthotropic shoots, low light.

	Shoot size (cm^2 $shoot^{-1}$)	**Leaves per shoot**	**Max leaf length (cm)**	**Max leaf width (cm)**	**Necrotic leaf surface (cm^2 $shoot^{-1}$)**	**Leaf growth (cm^2 $shoot^{-1}$ day^{-1})**	**Net shoot change (%)**
T1							
P_C	171.88 (34.71)	9.33 (0.67)	38.43 (9.47)	0.95 (0.03)	4.35 (3.40)	1.82 (0.21)	-1.42 (1.42)
O_C	166.48 (17.24)	5.26 (0.30)	54.31 (9.05)	0.94 (0.00)	0.33 (0.33)	1.38 (0.10)	
P_LL	134.87 (8.86)	8.33 (0.88)	31.67 (3.00)	0.90 (0.00)	2.85 (1.48)	0.91 (0.14)	-1.09 (1.09)
O_LL	145.84 (28.25)	4.69 (0.14)	42.42 (5.88)	0.90 (0.03)	0.26 (0.13)	0.82 (0.15)	
T2							
P_C	182.26 (13.51)	7.83 (1.83)	45.33 (5.95)	0.94 (0.02)	0.00 (0.00)	2.17 (0.27)	0.00 (0.00)
O_C	259.05 (37.14)	5.32 (0.24)	83.09 (12.10)	0.98 (0.01)	0.83 (0.49)	2.56 (0.21)	
P_LL	125.68 (12.15)	5.00 (0.58)	42.83 (6.22)	0.92 (0.02)	0.00 (0.00)	0.83 (0.05)	-2.74 (1.65)
O_LL	192.46 (26.93)	4.60 (0.40)	68.27 (1.99)	0.95 (0.03)	3.05 (1.15)	1.14 (0.14)	
T3							
P_C	154.73 (14.91)	6.67 (0.88)	41.50 (5.97)	0.95 (0.03)	0.00 (0.00)	1.40 (0.16)	-1.19 (1.19)
O_C	230.38 (8.55)	5.17 (0.33)	71.88 (6.46)	0.97 (0.00)	0.33 (0.17)	2.24 (0.14)	
P_LL	106.84 (11.14)	7.33 (1.20)	28.83 (1.17)	0.88 (0.02)	4.13 (4.13)	0.31 (0.10)	-5.19 (2.82)
O_LL	167.95 (31.73)	4.58 (0.36)	61.33 (9.88)	0.91 (0.04)	2.12 (1.77)	1.10 (0.12)	

Table 3.2 Results of one and two-way ANOVAs of morphological characteristics (shoot size, number of leaves per shoot, maximum leaf length and width, necrotic leaf surface), leaf growth rate and net shoot change at T1, T2 and T3. *P* <0.05 are in bold, *P* <0.1 are underlined.

ANOVA

T1		**Shoot size**		**Leaves per shoot**		**Max leaf length**		**Max leaf width**		**Necrotic surface**		**Leaf growth**	
Effect	**df**	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***
Shoot type	1	0.013	0.912	44.859	**0.000**	3.298	0.107	0.077	0.788	0.003	0.955	2.941	0.125
Light	1	1.397	0.271	1.852	0.211	1.618	0.239	5.205	0.052	2.924	0.126	22.615	**0.001**
ST×LL	1	0.113	0.746	0.139	0.719	0.122	0.736	0.006	0.941	0.000	0.983	1.286	0.290

T2		**Shoot size**		**Leaves per shoot**		**Max leaf length**		**Max leaf width**		**Necrotic surface**		**Leaf growth**	
Effect	**df**	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***
Shoot type	1	8.468	**0.020**	2.165	0.179	17.796	**0.003**	3.053	0.119	9.648	**0.015**	3.627	0.093
Light	1	6.232	**0.037**	3.230	0.110	1.338	0.281	1.859	0.210	3.144	0.114	54.671	**0.000**
ST×L	1	0.041	0.844	1.139	0.317	0.677	0.434	0.005	0.944	3.144	0.114	0.050	0.829

T3		**Shoot size**		**Leaves per shoot**		**Max leaf length**		**Max leaf width**		**Necrotic surface**		**Leaf growth**	
Effect	**df**	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***
Shoot type	1	13.115	**0.007**	7.327	**0.027**	22.433	**0.001**	1.029	0.340	1.186	0.308	39.693	**0.000**
Light	1	8.534	**0.019**	0.003	0.959	3.056	0.119	6.429	**0.035**	1.297	0.288	74.177	**0.000**
ST×L	1	0.148	0.710	0.634	0.449	0.026	0.877	0.029	0.870	0.087	0.775	0.028	0.872

Net shoot change	*T1*		*T2*		*T3*	
	df	**F**	***P***	**F**	***P***	**F**	***P***
Light	1	0.033	0.865	2.741	0.173	1.709	0.261

Photosynthetic parameters and pigment content

Multivariate analysis (2-way PERMANOVA) of photo-physiological variables (chlorophyll *a* fluorescence parameters and photosynthetic pigments) emphasized a significant effect of the low-light treatment at both sampling time points (i.e. after 15 and 30 days of exposure) (T1: $P_{(perm)} < 0.01$, T2: $P_{(perm)} < 0.001$; Table 3.3), whereas there were no differences in the photo-physiological response of plagiotropic *vs.* orthotropic shoots. This was confirmed by PCAs conducted at the same sampling times, in which most of the variance (84% in T1 and 73% in T2) was explained by the component 1 (PC1), which mainly clustered LL and control samples on the left and right side of the plot, respectively (Fig. 3.7 A, B). The behavior of horizontal and vertical shoots was similar; hence, they did not form well-distinct groups (Fig. 3.7 A, B).

Univariate analysis by two-way ANOVA showed no significant modification of dark-adapted chlorophyll fluorescence-derived photosynthetic parameters, i.e. basal fluorescence (F_0) and maximum quantum yield of PSII (Fv/Fm), in response to LL conditions or depending on the shoot type, neither after 15 (T1) nor 30 (T2) days of exposure (Fig. 3.8 and Table 3.4). On the contrary, most RLC-derived parameters were strongly affected by LL treatment. The effective photochemical efficiency (ΔF/Fm'), relative electron transport rate (r-ETR), and minimum saturating irradiance (Ik) were significantly reduced by 30 to 40% in LL plants, in respect to controls, both at T1 and T2, without any differences between plagiotropic and orthotropic shoots (T1: $P < 0.05$ for ΔF/Fm', $P < 0.01$ for r-ETR and Ik; T2: $P < 0.001$ for ΔF/Fm', r-ETR and Ik) (Fig. 3.8 and Table 3.4). The maximum photo-protective capacity of thermal energy dissipation by non-photochemical quenching (NPQ) was similar across LL and control plants, and between different shoot types (Fig. 3.8 and Table 3.4). Leaf pigment content (Chl*a*, Chl*b* and carotenoids) did not vary during the exposure to LL or with shoot type, at any sampling time (Fig. 3.9 and Table 3.4). The lowest pigment content was recorded in leaves of apical shoots after 30 days of exposure (T2) (Fig. 3.9), although such variations were not statistically significant. Mean values of photosynthetic parameters and pigment concentrations determined at T1 and T2, in control and LL exposed plants, can be retrieved from Table A3.2 in Appendix III.

Table 3.3 Results of 2-way PERMANOVAs conducted on multivariate photo-physiological variables (photosynthetic parameters and pigment content), at T1 and T2. $P_{(perm)}$ <0.05 are in bold.

2-way PERMANOVA				
Photo-physiology				
T1				
Source	**df**	**Pseudo-F**	$P_{(perm)}$	**Unique perms**
Shoot type	1	0.066	0.9643	8845
Light	1	11.395	**0.0032**	8876
ST×L	1	0.31997	0.7848	8902
T2				
Source	**df**	**Pseudo-F**	$P_{(perm)}$	**Unique perms**
Shoot type	1	0.35103	0.7951	8923
Light	1	14.464	**0.0023**	8923
ST×L	1	0.46634	0.7071	8893

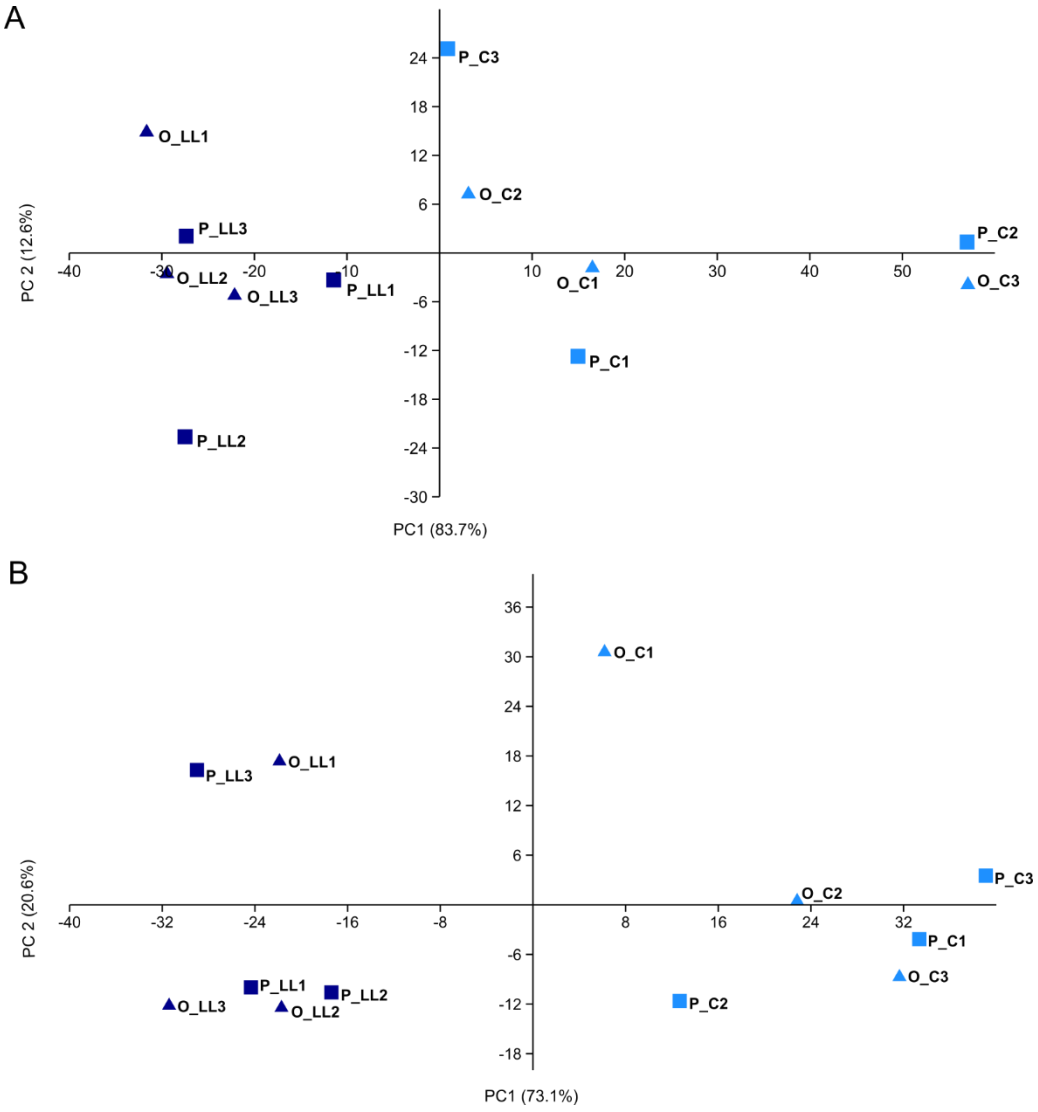

Fig. 3.7 PCAs conducted on (A) all photo-physiological variables (photosynthetic parameters and pigment content) at T1; and (B) all photo-physiological variables (photosynthetic parameters and pigment content) at T2. Different colors refer to different treatments (light blue = control, C; dark blue = low light, LL). Different symbols refer to different shoot types (filled triangles = orthotropic, O; filled square = plagiotropic, P). Numbers refer to the different replicates considered for the analysis (*n*=3).

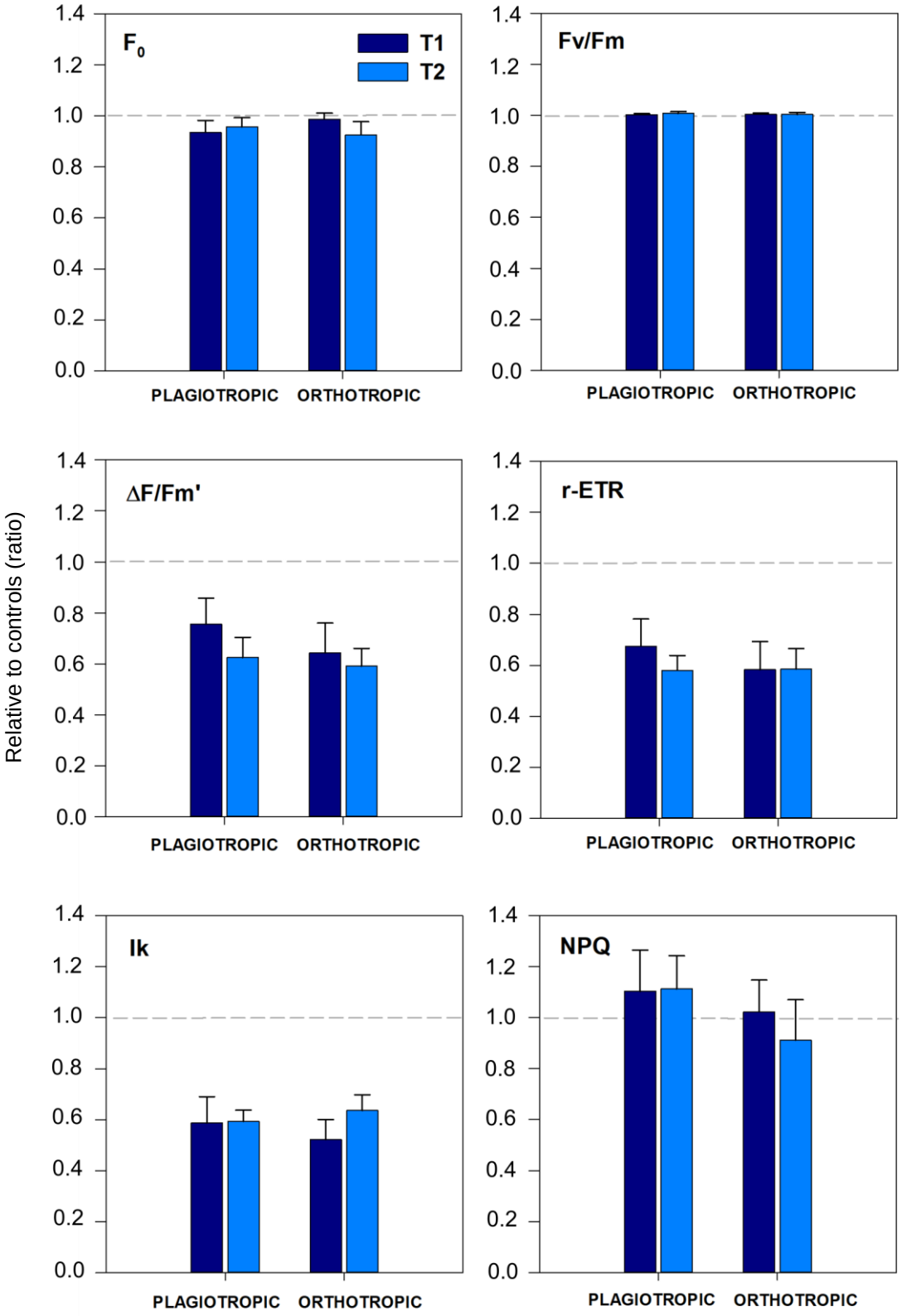

Fig. 3.8 Changes in photosynthetic parameters in LL relative to control plants (ratio). F_0, basal fluorescence; Fv/Fm, maximum photochemical efficiency of PSII; ΔF/Fm', effective quantum yield; r-ETR max, relative maximum electron transport rate; Ik, minimum saturating irradiance; NPQ, non-photochemical quenching. Values above or below the dashed grey line indicate an increase or decrease in respect to controls, respectively. Data are mean ± SE (*n*=3). Results of 2-way ANOVAs for each sampling time are reported in Table 3.4.

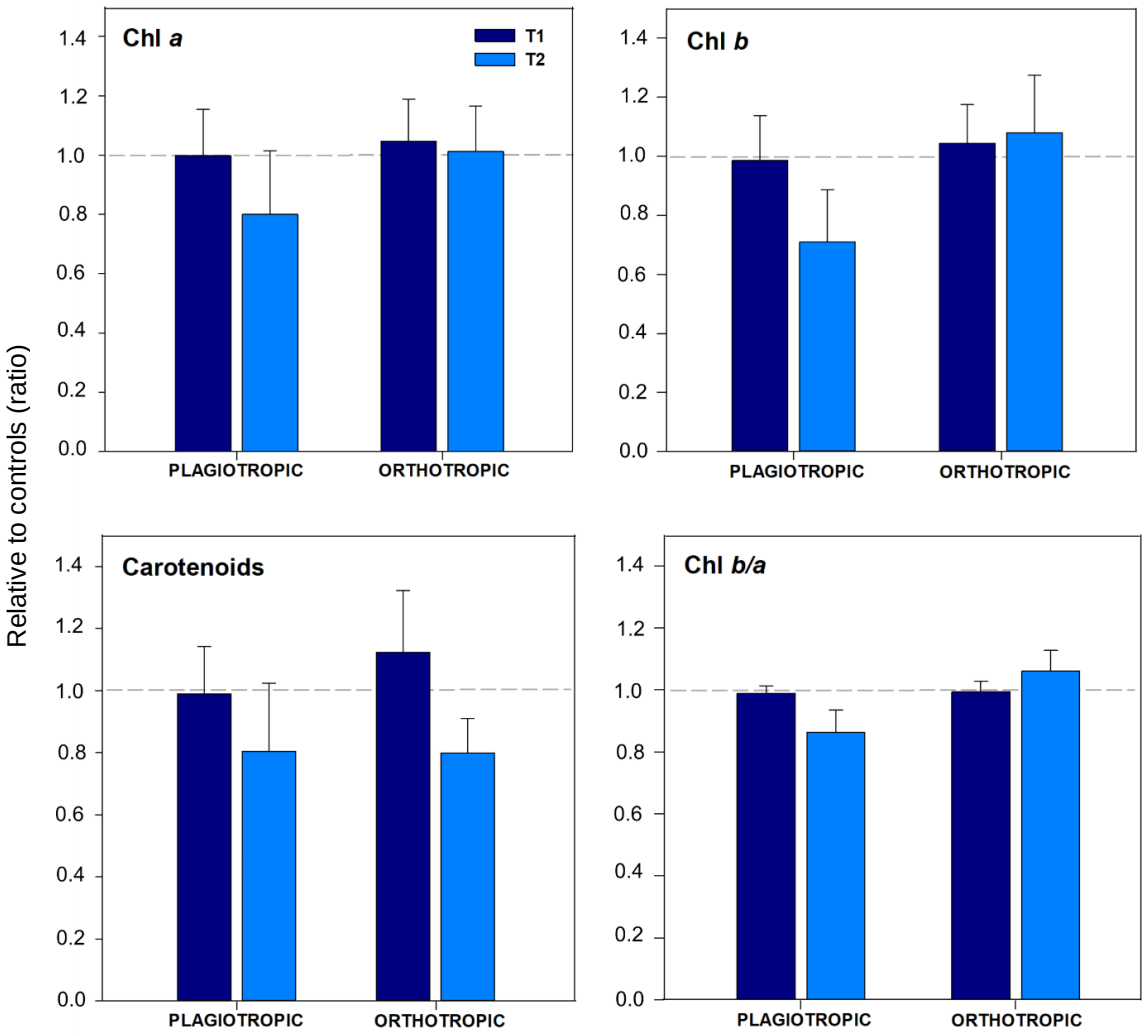

Fig. 3.9 Changes in chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), total carotenoids concentrations and Chl *b/a* molar ratio, in LL relative to control plants (ratio). Values above or below the dashed grey line indicate an increase or decrease in respect to controls, respectively. Data are mean ± SE (*n*=3). Results of 2-way ANOVAs for each sampling time are reported in Table 3.4.

Table 3.4 Results of two-way ANOVAs to assess the effect of shoot type (ST) and light (L) treatment on individual photo-physiological variables (photosynthetic parameters and pigment content), at T1 and T2. *P* <0.05 are in bold, *P* <0.1 are underlined.

Two-way ANOVA

T1		F_0		F_v/F_m		ΔF/Fm'		r-ETR		Ik		NPQ	
Effect	**df**	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***
Shoot type	1	0.183	0.680	2.327	0.166	0.208	0.660	0.769	0.406	0.028	0.872	2.134	0.182
Light	1	2.093	0.186	1.905	0.205	8.121	**0.021**	16.752	**0.003**	16.758	**0.003**	0.401	0.544
ST×L	1	0.923	0.365	0.047	0.833	0.297	0.601	0.384	0.553	0.113	0.746	0.182	0.681
		Chl *a*		**Chl *b***		**Carotenoids**		**Chl *b*/*a***					
		F	***P***	**F**	***P***	**F**	***P***	**F**	***P***				
Shoot type	1	0.000	0.997	0.010	0.921	0.059	0.815	0.200	0.666				
Light	1	0.045	0.838	0.018	0.896	0.201	0.666	0.133	0.725				
ST×L	1	0.054	0.821	0.081	0.783	0.287	0.607	0.061	0.811				

T2		F_0		F_v/F_m		ΔF/Fm'		r-ETR		Ik		NPQ	
Effect	**df**	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***
Shoot type	1	0.161	0.698	0.000	0.987	2.066	0.189	1.107	0.324	0.547	0.481	0.086	0.777
Light	1	3.269	0.108	2.936	0.125	32.771	**0.000**	38.029	**0.000**	50.298	**0.000**	0.003	0.961
ST×L	1	0.252	0.629	0.290	0.605	0.001	0.972	0.009	0.926	0.445	0.524	0.905	0.369
		Chl *a*		**Chl *b***		**Carotenoids**		**Chl *b*/*a***					
		F	***P***	**F**	***P***	**F**	***P***	**F**	***P***				
Shoot type	1	0.496	0.501	0.198	0.668	1.385	0.273	1.560	0.247				
Light	1	0.511	0.495	0.871	0.378	1.931	0.202	1.183	0.308				
ST×L	1	0.621	0.454	2.039	0.191	0.012	0.916	4.284	0.072				

3.3.2 Transcriptomic responses of leaves and SAMs of plagiotropic and orthotropic P. oceanica shoots to low light

Transcriptome sequencing, de-novo assembly and functional annotation

Twenty-four cDNA libraries obtained from two different organs and shoot types, were used for the assembly of a new *P. oceanica* transcriptome. The Illumina sequencing generated 498,017,114 single-end reads (average length = 75 bp), with a mean per-base quality beyond 36 ("very good quality"). Raw reads were quality-trimmed to obtain a final amount of 421,487,471 HQ reads (84.63% of initial raw reads) and provided as input to Trinity software for the *de-novo* assembly (Table A3.1). To achieve the most comprehensive transcriptome as possible, this new assembly was merged with three previously obtained *P. oceanica* transcriptomes, derived from leaves collected at different depth and time of the day (D'Esposito et al. 2017), heat-stressed leaves (Marín-Guirao et al. 2017), as well as female and male flower sections (Entrambasaguas et al. 2017). This combined transcriptome consisted of 281,925 transcripts (≥ 200 bp), with an overall size of 156,519,687 bases (156.5 Mb), a mean length of 993 bp (spanning from 201 to 17,138) and N50 = 1,964 (Table 3.5). Transcriptome GC composition was 41% (Table 3.5). Approximately 108,000 contigs (ca. 38%) were over 1,000 bp (1Kb) long. On average, 80.3% of the reads mapped back to the assembly indicating that the assembled transcriptome represented most of sequenced reads. From this merged transcriptome, the longest isoforms for each transcript were selected to support subsequent analysis (166,231 transcripts).

Table 3.5 Summary statistics of the combined *P. oceanica* transcriptome. % GC = the proportion of guanidine and cytosine nucleotides among total nucleotides; N50 = the length of the longest contig such that all contigs of at least that length compose at least 50% of the bases of the assembly.

Basic statistics of the merged *P. oceanica* transcriptome	
Total number of transcripts	281,925
Number of Trinity "genes"	157,553
GC (%)	41.07
Mean (bp)	993.44
Minimum contig length (bp)	204
Maximum contig length (bp)	17,138
N50 value	1,964
Total assembled bases	156,519,687

A total of 167,446 transcripts (59.39%) significantly matched to known proteins, leaving 40.61% of the sequences without a significant matching (Table 3.6 and Fig. 3.10). Most of successfully annotated transcripts matched to the plant species *Asparagus officinalis* (77,316 blast hits) and *Vitis vinifera* (72,900 blast hits), but the species with the highest number of top blast hits (lowest e-value matching) was the seagrass *Z. marina* for which the genome is available (Olsen et al. 2016) (8,685 blast hits). The annotation process also retrieved information on Gene Ontology (GO) terms (as biological processes, molecular functions and cellular components), KEGG pathways and enzyme codes. Based on sequence homology, 140,569 sequences (49,86% of the total transcripts) were successfully assigned to at least one GO term (Table 3.6); top 20 GO term distribution as BP, MF and CC can be retrieved from Fig. A3.2 in Appendix III. KEGG annotation was obtained for 8,398 transcripts (3% of total transcripts), whereas 46,495 transcripts (16.5% of total transcripts) retrieved a significant enzyme code annotation (Table 3.6); of these, 42.4% were annotated as "Hydrolases", 40% as "Transferases", followed by "Oxidoreductases" (13.5%) (Fig A3.1 in Appendix III for full EC annotation).

Table 3.6 Summary of annotation results for the combined *P. oceanica* transcriptome.

Functional annotation of the merged *P. oceanica* transcriptome	
	Number of sequences
Blast hits	26,877
GO mapping	31,061
B2G annotation	109,508
KEGG annotation	8,398
Enzyme code (EC)	46,495

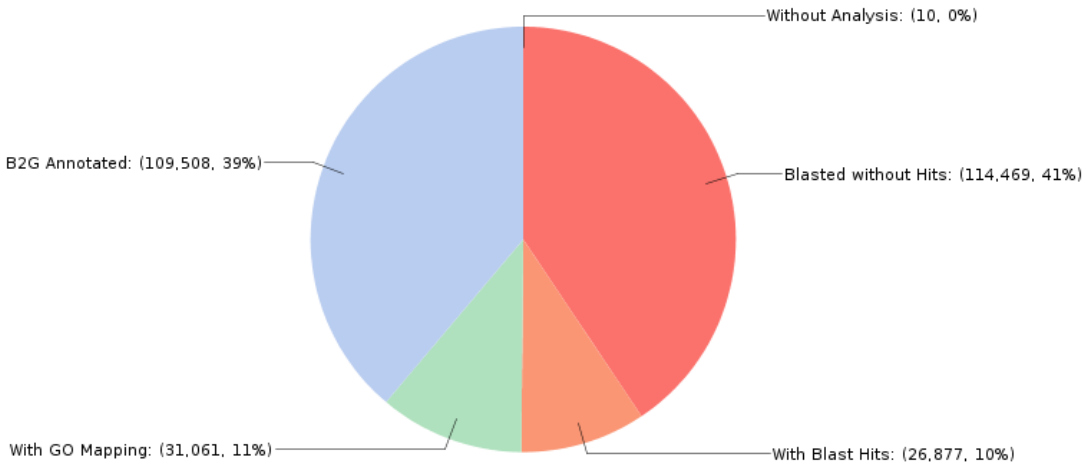

Fig. 3.10 Pie chart of functional annotation for the merged *P. oceanica* transcriptome.

Differential gene-expression analysis

Differential gene-expression (DEG) analysis was conducted for each plant organ (leaf and SAM), separately, or comparing directly the two organs (leaf *vs.* SAM), under control and LL conditions, and for the two different shoot types (plagiotropic and orthotropic). A summary of the pairwise comparisons considered in this study and the number of significantly (up or down) differentially expressed genes (DEGs) for each comparison (FDR <0.05; FC < ±2), can be retrieved from the Table 3.7:

Table 3.7 Summary of DEG analyses considered in this study with relative number of significantly up- or down-regulated genes. Contrasts analyzed in this chapter are in bold.

Pairwise comparison	**DEGs** (FDR < 0.05; FC > ±2)	
LEAF	**UP**	**DOWN**
P_LL - P_C	132	191
O_LL - O_C	174	58
SAM		
MP_C - MO_C	6	-
MP_LL - MO_LL	19	60
MP_LL - MP_C	247	656
MO_LL - MO_C	179	225
LEAF vs. *SAM*		
P_C - MP_C	2449	2771
P_LL - MP_LL	1895	2196
O_C - MO_C	2626	2743
O_LL - MO_LL	1801	2360

P_LL = leaf of plagiotropic shoot, low light; P_C = leaf of plagiotropic shoot, control light; O_LL = leaf of orthotropic shoot, low light; O_C = leaf of orthotropic shoot, control light; MP_C = SAM of plagiotropic shoot, control light; MP_LL = SAM of plagiotropic shoot, low light; MO_C = SAM of orthotropic shoot, control light; MO_LL = SAM of orthotropic shoot, low light.

DEG analyses revealed significantly different gene-expression patterns primarily between the two selected organs (leaf *vs.* SAM), as demonstrated by the higher number of DEGs identified in these comparisons (5220, 4091, 5369 and 4161 DEGs in P_C-MP_C, P_LL-MP_LL, O_C-MO_C and O_LL-MO_LL, respectively) (Table 3.7). The factor "light" was less important than the "organ type" in modulating the transcriptomic response, but still a relevant number of DEGs was identified within the C *vs*. LL contrasts (323, 232, 903 and 404 DEGs in P_LL-P_C, O_LL-O_C, MP_LL-MP_C and MO_LL-MO_C, respectively)

(Table 3.7). Only minor differences were encountered between SAMs of plagiotropic *vs.* orthotropic shoots, with a total of 6 and 79 DEGs for MP_C-MO_C and MP_LL-MO_LL, respectively (Table 3.7). Differences between leaf tissues of plagiotropic *vs.* orthotropic shoots were not directly explored in this study.

The relative contribution of the factors "light" and "organ type" on the differential patterns of gene expression is clearly visible from the PCA (Fig. 3.11 A). Specifically, the PC1 explained most of the total variance (46.23%), and segregated two well-distinct sample groups, corresponding to leaves (on the left side of the plot) and apical meristems (on the right side of the plot). Vertical segregation along the PC2 occurred between LL and control samples (5.91% total variance). Accordingly, all LL samples (both leaves and SAMs) clustered at the bottom of the plot, whereas control samples were represented in the upper part of the plot (Fig. 3.11 A).

Interestingly, LL exposure had a greater effect on the transcriptomic response of SAMs, rather than leaves, as revealed by the larger number of DEGs identified in the contrasts MP_LL-MP_C and MO_LL-MO_C (903 and 404 DEGs, respectively), in respect to P_LL-P_C and O_LL-O_C (323 and 232 DEGs, respectively) (Table 3.7). This was also evident from the PCA, where a greater separation between control and LL meristem samples, compared to control and LL leaves, was visible (Fig. 3.11 A). Finally, it is worth noticing that in the C *vs.* LL comparisons of both SAMs and leaves, a higher number of DEGs was always identified for plagiotropic shoots (323 and 903 for P_LL-P_C and MP_LL-MP_C, respectively), in respect to orthotropic ones (232 and 404 for O_LL-O_C and MO_LL-O_C, respectively) (Table 3.7). The profile of expression across different samples at gene level was also examined through a hierarchical clustering (Fig. 3.11 B). A clear differentiation was present between leaf and SAM samples, where most DEGs were up-regulated in one organ and down-regulated in the other, or vice-versa. Four major clusters of expression (indicated with numbers 1 to 4 in Fig. 3.11 B) were noticeable: the first one grouping genes down-expressed in meristems and LL leaves, and up-regulated only in control leaves; the second and third clusters comprising genes largely down-regulated in the meristem in respect to leaves, and vice-versa; the last one grouping genes with a mixed behavior, with a subset showing a similar pattern in LL *vs.* C regardless the organ type (yellow rectangle) (Fig. 3.11 B).

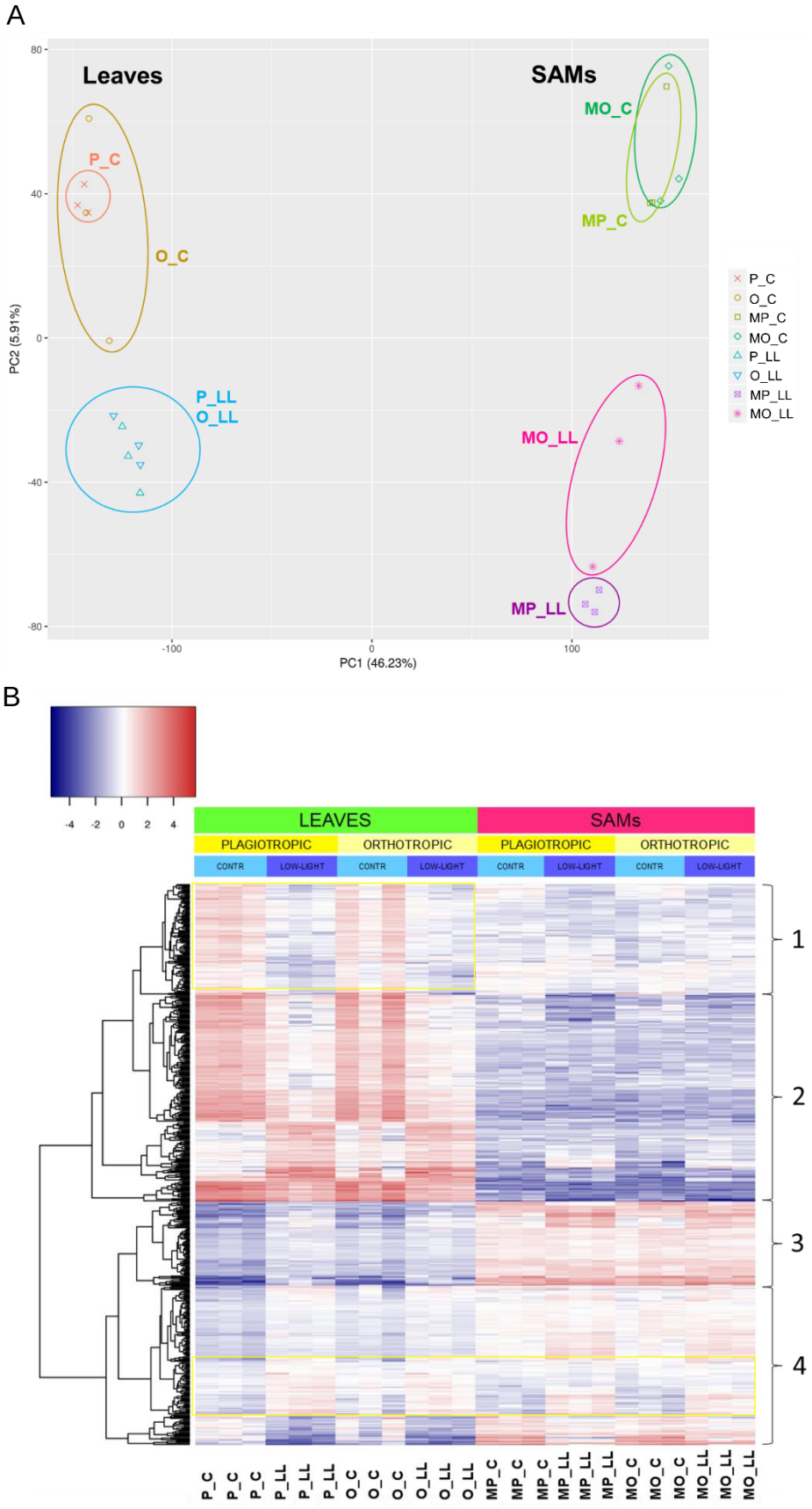

Fig. 3.11 (A) PCA based on expression values of the different biological replicates of P-O leaves and SAMs, in control and LL conditions; (B) Heatmap of hierarchical cluster analysis of gene-expression patterns in the same 24 samples. Deeper colors indicate higher up (red) or down (blue) regulation.

For the purpose of this chapter, only 6 pair-wise comparisons have been deeply analyzed, namely the direct contrast MP *vs.* MO, in C and LL conditions, separately (MP_C-MO_C and MP_LL-MO_LL), and the direct contrast C *vs.* LL in SAMs (MP_LL-MP_C and MO_LL-MO_C), and leaves (P_LL-P_C and O_LL-O_C). Explored comparisons are indicated in bold in Table 3.7.

Meristem-specific transcriptomic response in plagiotropic vs. orthotropic shoots

In order to identify putative molecular properties underlying physiological differences between meristem tissues in apical *vs.* vertical shoots, DEG analysis was conducted for each light conditions, separately. Interestingly, only few DEGs were identified under control light (MP_C-MO_C), whereas under LL (MP_LL-MO_LL), such differences were amplified. Transcripts encoding for some photosynthetic components and chlorophyll *a/b* binding (CAB) proteins were among the few DEGs in the meristem of plagiotropic (MP) *vs.* orthotropic (MO) shoots, in control light. Up-regulated genes included two subunits of PSI (*PSI-D2* and *PSI-O*), and three CAB proteins (*CAB-21*, *CAB-8* and *CAB-6A*). Accordingly, the two most significant GO enriched biological processes (BP) were *photosynthesis and light harvesting in PSI* and *protein-chromophore linkage* (see Table 3.8 for full GO-BP list). No down-regulated genes were identified in this contrast.

Under LL, it was observed an enrichment for transcripts included in 15 GO (BP) categories such as *phyllome development, DNA damage response, response to light stimulus, response to lipid* and *cell division* (see Table 3.8 for full GO-BP list). Most DEGs falling in these categories were up-regulated in MO (down-regulated in MP) (60), whereas only 19 were over-expressed in MP (down-regulated in MO) (Table 3.7). Up-regulated functions in MO_LL included many genes involved in lipid metabolism, lipid-catabolic processes and transport (e.g. *OMEGA-3 FATTY ACID DESATURASE, STEAROYL-[ACYL-CARRIER-PROTEIN] 9-DESATURASE 6, GDSL ESTERASE/LIPASE, PHOSPHOLIPASE A2-ALPHA, LIPID-TRANSFER PROTEIN DIR1, NON-SPECIFIC LIPID-TRANSFER PROTEIN*). Equally up-regulated were genes involved in cell-wall organization (*XYLOGLUCAN ENDOTRANS GLUCOSYLASE/HYDROLASE PROTEIN 9, ALTERED XYLOGLUCAN 4*), degradation (*POLYGALACTURONASE*), and trehalose catabolism (*ALPHA, ALPHA-TREHALASE*). A transcript for a carbohydrate transporter (*NUCLEOTIDE-SUGAR TRANSPORTER 2*), and *NITRATE TRANSPORTER 1.1* (*NRT1*), which is responsible for the root-to-shoot bidirectional nitrate translocation, were also more expressed in orthotropic shoots, in respect to plagiotropic ones. Interestingly, two transcripts encoding for RNA-binding proteins involved in DNA damage response, cell-cycle arrest and

cell death were identified, namely *RNA-BINDING PROTEIN 24* (*RBM24*), *ABA-REGULATED RNA-BINDING PROTEIN 1* (*ARP1*). Within the GO term *response to light stimulus*, three remarkable transcripts were recognized: *GATA TRANSCRIPTION FACTOR 12* (*GAT12*), which is involved in the regulation of some light-responsive genes and acts as a transcription activator involved in xylem formation, the *POLLEN-SPECIFIC PROTEIN SF21* (*SF21*) and the protein *TEMPRANILLO 1* (*TEM 1*), which is a transcriptional repressor of flowering.

Among the top up-regulated transcripts in MP_LL were the chaperone protein *DNAJ*, the transcription factor *GHD7*, which also plays a major role as a repressor of flowering, and the CAB proteins 21 and 6A.

Table 3.8 Full list of GO enriched BP (FDR <0.05) in MP_C-MO_C and MP_LL-MO_LL. GO identifiers, GO-BP names and FDR values, are given.

GO ID	GO – Biological process	FDR
MP_C-MO_C		
0009768	photosynthesis, light harvesting in photosystem I	9.77E-09
0018298	protein-chromophore linkage	2.79E-05
0009645	response to low light intensity stimulus	2.16E-03
0010218	response to far red light	2.72E-02
0010114	response to red light	2.75E-02
0009644	response to high light intensity	3.61E-02
MP_LL-MO_LL		
0048827	phyllome development	7.58E-03
0009753	response to jasmonic acid	9.51E-03
0046916	cellular transition metal ion homeostasis	1.32E-02
0051924	regulation of calcium ion transport	1.58E-02
0042446	hormone biosynthetic process	1.58E-02
0009627	systemic acquired resistance	1.59E-02
0006978	DNA damage response, signal transduction by p53 class mediator resulting in transcription of p21 class mediator	2.17E-02
0009416	response to light stimulus	2.51E-02
0070935	3'-UTR-mediated mRNA stabilization	2.57E-02
0045787	positive regulation of cell cycle	3.74E-02
0033993	response to lipid	4.19E-02
0090696	post-embryonic plant organ development	4.30E-02
1901576	organic substance biosynthetic process	4.65E-02
0009751	response to salicylic acid	4.84E-02
0051301	cell division	4.93E-02

Leaf response of plagiotropic and orthotropic shoots in control vs. LL conditions

DEG analysis revealed a slightly higher number of DEGs in plagiotropic shoots exposed to LL (P_LL-P_C; 323), in respect to orthotropic ones (O_LL-O_C; 232) (Table 3.7). Surprisingly, few DEGs were shared between the two contrasts (76), whereas the most part of them were exclusively associated to the LL response of apical (248) or vertical shoots (157) (see Venn diagram in Fig. 3.12).

The differential response to LL between the two shoot types was further supported by the GO enrichment analysis. Even though a reduced total number of DEGs was identified in the contrast O_LL-O_C in respect to P_LL-P_C, a substantial higher number of enriched GO terms was associated with the former contrast (Table 3.9). With a total of 36 GO-BP terms, the transcriptomic reprogramming observed in the leaf tissue of vertical shoots under LL appeared to be much more complex and multifaceted than that of apical shoots, which was restricted to a total of 13 GO-BP enriched terms (see Figs. 3.13 and 3.14 and Table 3.9 for full list). As for DEGs, few enriched biological functions were in common between the two contrasts, while the large part of them was specifically associated with the response of plagiotropic or orthotropic shoots (Table 3.9).

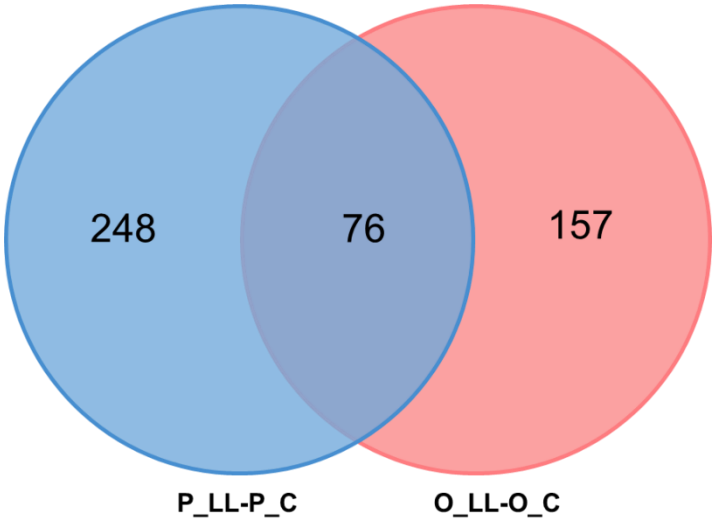

Fig. 3.12 Venn diagram depicting shared and unique DEGs in the contrasts P_LL-P_C and O_LL-O_C.

Shared response of plagiotropic and orthotropic leaves under LL: Among the few shared DEGs identified between P_LL-P_C and O_LL-O_C, it is worth noticing the presence of transcripts involved in key plant metabolic processes, such as photosynthesis (*PHOTOSYSTEM II 22 KDA PROTEIN*), chlorophyll biosynthesis (*MAGNESIUM-PROTOPORPHYRIN IX MONOMETHYL ESTER*), and glycolysis/gluconeogenesis

(*FRUCTOSE-BISPHOSPHATE ALDOLASE 1*), as significantly down-regulated under LL. Similarly, some transcripts encoding for amino-acid, oligopeptide and nitrate transporters (*AMINO-ACID PERMEASE 2*, *OLIGOPEPTIDE TRANSPORTER 3* and *4*, and *NRT 1.1*) were among top down-regulated genes. Shared up-regulated genes included some transcripts for stress-related proteins (e.g. *PEROXIDASE 45* and *UNIVERSAL STRESS PROTEIN A*), and interestingly *GALACTINOL-SUCROSE GALACTOSYLTRANSFERASE 6*, which is known to be induced by dark.

LL response of plagiotropic leaves: GO enriched BP in P_LL-P_C included plant hormone-related signaling pathways and response to plant hormones (*negative regulation of cytokinin-activated signaling pathway* and *response to abscisic acid*), and secondary metabolite-related metabolic processes (*pigment biosynthetic process*, *regulation of phenylpropanoid metabolism*, *flavonoid metabolic process* and *geranylgeranyl diphosphate biosynthetic process*) (Fig. 3.13 and Table 3.9). Most transcripts specifically associated with hormone signaling pathways were transcriptional factors, and were generally up-regulated in LL. In addition, hormone receptors like *ABSCISIC ACID RECEPTOR PYL8* and some genes related to the auxin-activated signaling pathway (e.g. *DORMANCY-ASSOCIATED PROTEIN 1* and *AUXIN EFFLUX CARRIER 8*) were also over-expressed. The same was observed for proteins involved in secondary metabolite biosynthesis, such as terpenes and anthocyanins (e.g. *GERANYLGERANYL PYROPHOSPHATE SYNTHASE* and *ANTHOCYANIDIN 3-O-GLUCOSYLTRANSFERASE 5*). Among down-regulated genes in P_LL-P_C, it is worth remarking the presence of two transcripts involved in phototropism and photoperiodism (*ROOT PHOTOTROPISM PROTEIN 2* and *PHYTOCHROME A-ASSOCIATED F-BOX PROTEIN*), and of the enzymes *NITRATE REDUCTASE*, involved in the first step of nitrate assimilation, and *SUCROSE PHOSPHATE SYNTHASE 4*, which plays a fundamental role in photosynthetic sucrose synthesis.

Notably, only in the comparison P_LL-P_C, 15 transcripts associated to retroelements (retrotransposons and retroviruses) were identified as differentially expressed, and they were mostly down-regulated under LL.

LL response of orthotropic leaves: As commented above, a higher number of GO enriched processes were recognized for the contrast O_LL-O_C. Among these, the most significant ones (FDR <0.01) were *cellular protein modification processes*, *regulation of transcription*, *oligopeptide transport*, *cellular response to stress* and *negative regulation of cytokinin-activated signaling pathway*" (Fig. 3.14 and Table 3.9). Several other GO-BP were enriched under LL at FDR <0.05, for example *amino-acid import*, *phloem nitrate loading*, *seed development*, *developmental growth involved in morphogenesis*, *organic hydroxy compound*

metabolic process, *positive regulation of proteolysis*, *cellular component biogenesis*, *secondary metabolic process*, *regulation of response to external stimulus* and *steroid metabolic process* (Fig. 3.14; see Table 3.9 for full list).

Overall, transcripts involved in phloem nitrate loading, oligopeptide/amino acid transport, as well as carbohydrate transport, were down-regulated in LL (e.g. *OLIGOPEPTIDE TRANSPORTER 7*, *NRT 1.10*, *AMINO ACID PERMEASE 3*, *INOSITOL TRANSPORTER 4*). Up-regulated transcripts were mostly included in the GO categories *regulation of transcription*, *cellular protein modification*, *cellular stress response* and *response to ethylene*. Several transcripts with a role in protein repair were found over-expressed in LL, including many chaperones and chaperone regulators (e.g. *HSP70-HSP90 ORGANIZING PROTEIN 1* and *BAG FAMILY MOLECULAR CHAPERONE REGULATOR 5*), members of the universal stress and LEA protein families, as well as some proteins involved in DNA damage response (e.g. *NON-STRUCTURAL MAINTENANCE OF CHROMOSOMES ELEMENT* 1 and 4, and *HUS1*). Lastly, the enzyme *SUCROSE SYNTHASE 4*, a fundamental sucrose-cleaving enzyme, was found among over-expressed transcript in LL.

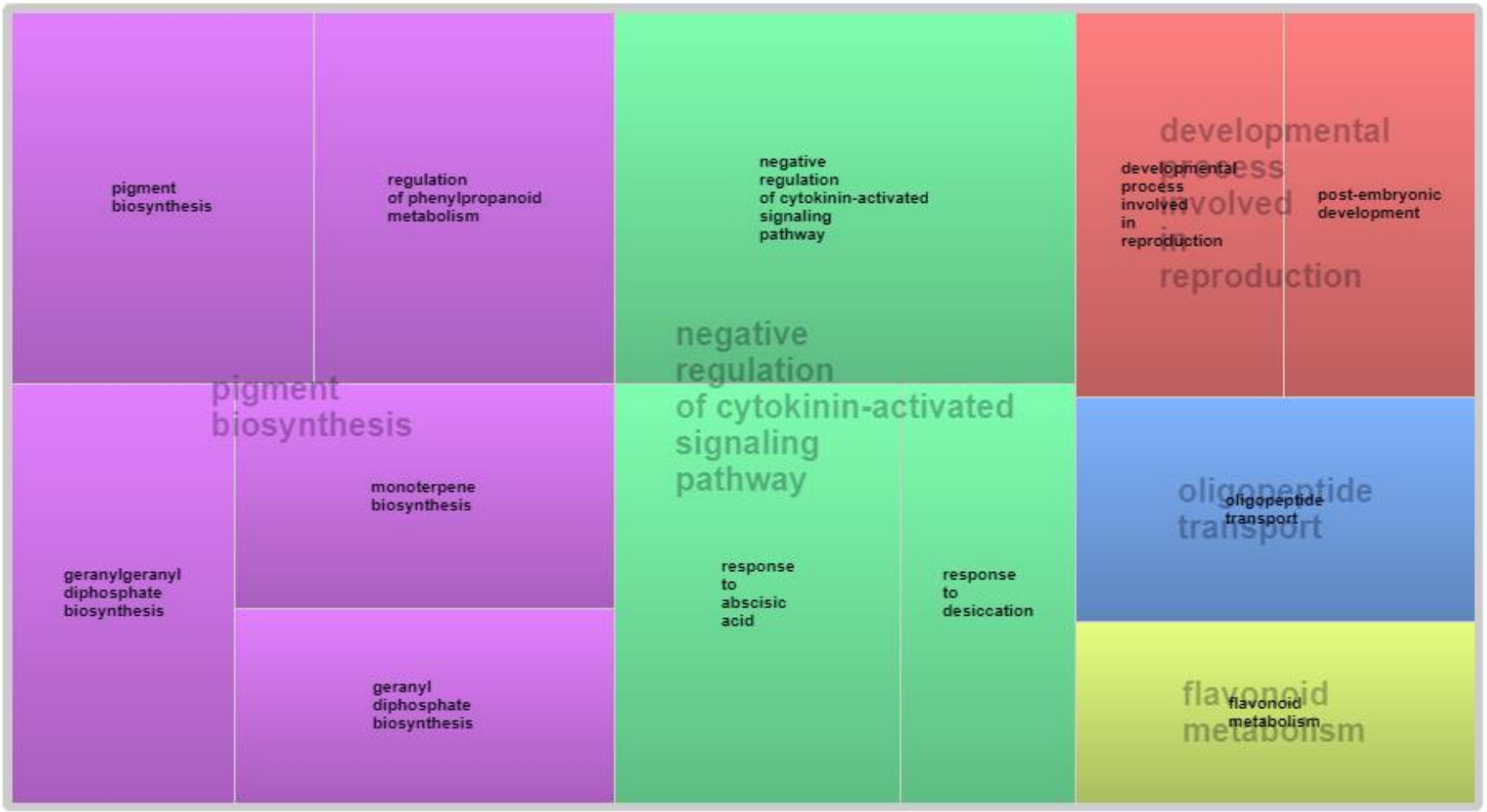

Fig. 3.13 TreeMap representation of GO enriched BP (FDR <0.05) in the contrast P_LL-P_C. GO terms are colored by semantic similarity and size reflects the abs_log10_pvalue.

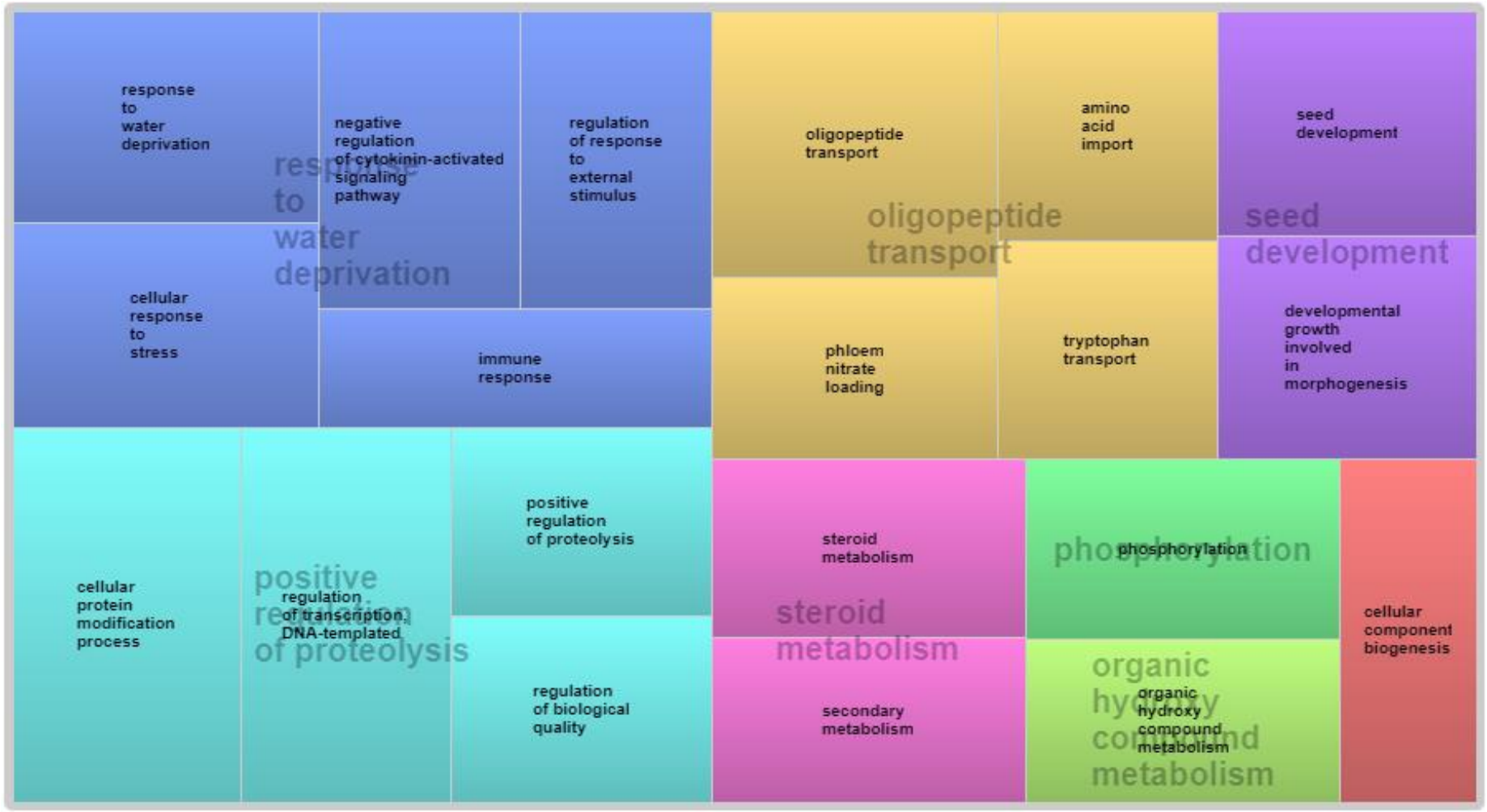

Fig. 3.14 TreeMap representation of GO enriched BP (FDR <0.05) in the contrast O_LL-O_C. GO terms are colored by semantic similarity and size reflects the abs_log10_pvalue.

Table 3.9 Full list of GO enriched BP (FDR <0.05) in P_LL-P_C and O_LL-O_C. GO identifiers, BP names and FDR values are given. Shared BP are in bold.

GO ID	GO – Biological process	FDR
P_LL-P_C		
0080037	**negative regulation of cytokinin-activated signaling pathway**	3.31E-06
0009737	response to abscisic acid	5.52E-04
0046148	pigment biosynthetic process	1.13E-03
2000762	regulation of phenylpropanoid metabolic process	1.28E-03
0033386	geranylgeranyl diphosphate biosynthetic process	6.78E-03
0006857	**oligopeptide transport**	9.24E-03
0015698	inorganic anion transport	1.23E-02
0043693	monoterpene biosynthetic process	1.40E-02
0003006	developmental process involved in reproduction	2.22E-02
0033384	geranyl diphosphate biosynthetic process	4.20E-02
0009791	post-embryonic development	4.43E-02
0009269	response to desiccation	4.53E-02
0009812	flavonoid metabolic process	4.86E-02
O_LL-O_C		
0006464	cellular protein modification process	2.58E-04
0006355	regulation of transcription, DNA-templated	7.44E-04
0006857	**oligopeptide transport**	9.75E-04
0009414	response to water deprivation	4.27E-03
0033554	cellular response to stress	6.28E-03
0080037	**negative regulation of cytokinin-activated signaling pathway**	9.12E-03
0048316	seed development	1.12E-02
0060560	developmental growth involved in morphogenesis	1.18E-02
0032101	regulation of response to external stimulus	1.29E-02
0016310	phosphorylation	1.46E-02
0008202	steroid metabolic process	1.50E-02
0006970	response to osmotic stress	1.85E-02
0048588	developmental cell growth	1.89E-02
0090408	phloem nitrate loading	2.53E-02
0031347	regulation of defense response	2.67E-02
0019748	secondary metabolic process	2.67E-02
0009723	response to ethylene	2.74E-02
1901615	organic hydroxy compound metabolic process	2.81E-02
0043090	amino acid import	2.95E-02
0048437	floral organ development	3.07E-02
2000026	regulation of multicellular organismal development	3.18E-02
0035524	proline transmembrane transport	3.18E-02
0045862	positive regulation of proteolysis	3.55E-02
0051254	positive regulation of RNA metabolic process	3.61E-02
0065008	regulation of biological quality	3.64E-02
0015810	aspartate transmembrane transport	3.87E-02
0010628	positive regulation of gene expression	3.87E-02
0006868	glutamine transport	4.11E-02
0048235	pollen sperm cell differentiation	4.11E-02
0015827	tryptophan transport	4.11E-02
0048545	response to steroid hormone	4.27E-02
0015825	L-serine transport	4.34E-02
0009556	microsporogenesis	4.50E-02
0044085	cellular component biogenesis	4.71E-02
0006955	immune response	4.89E-02
0006996	organelle organization	4.93E-02

Meristem response of plagiotropic and orthotropic shoots in control vs. LL conditions

Similarly to what observed in the analysis of leaf tissues, a higher number of DEGs was recognized in the SAM of plagiotropic rather than orthotropic shoots under LL (MP_LL - MP_C, 903; MO_LL - MO_C, 404) (Table 3.7). A total of 210 DEGs were in common between the two contrasts, whereas 694 were exclusively associated to plagiotropic shoots, and 195 with orthotropic ones (see Venn diagram in Fig. 3.15).

The differential response to LL between the two shoot types was supported also in this case by the GO enrichment analysis. Even though a reduced total number of DEGs was identified in MO_LL-MO_C, in respect to MP_LL-MP_C, a considerable higher number of enriched biological processes was associated with the former contrast, in respect to the latter (Tables 3.10 and 3.11).Specifically, a total of 108 enriched GO-BP (FDR <0.05) were identified in MP_LL-MP_C, in respect to the 283 GO-BP (FDR <0.05) found for MO_LL-MO_C. For simplicity, only biological processes enriched at FDR <0.01 are reported in Tables 3.10 and 3.11; GO-BP subsets are also depicted in Figs. 3.16 and 3.17.

A total of 14 GO enriched biological functions (FDR <0.01) were shared between the two contrasts, where the remaining part was specifically associated with the response of plagiotropic or orthotropic shoots (see Tables 3.10 and 3.11).

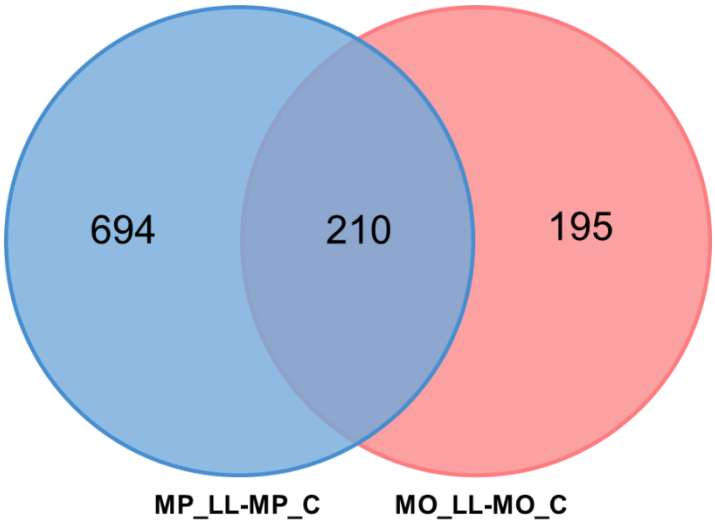

Fig. 3.15 Venn diagram depicting shared and unique DEGs in the contrasts MP_LL-MP_C and MO_LL-MO_C.

Shared response of plagiotropic and orthotropic SAMs under LL: Surprisingly, many structural and functional components involved in the photosynthetic process, chlorophyll biosynthesis and photosynthetic carbon-assimilation pathways, as well as members of light harvesting complexes, were identified as differentially expressed in the transcriptome analysis of SAMs. Yet, the number of DEGs related to abovementioned processes was

actually higher in SAMs rather than leaves. The vast majority of these DEGs were strongly down-regulated under LL in both plagiotropic and orthotropic shoots (see Table A3.3 in Appendix III). Among down-regulated transcripts were photosystem subunits (e.g. *PSI SUBUNIT O* and *III, PSII REACTION CENTER W*), electron transport-related proteins (e.g. *NAD(P)H-QUINONE OXIDOREDUCTASE CHAIN 4, PHOTOSYNTHETIC NDH SUBUNIT OF SUBCOMPLEX B4*), and proteins assisting photosystem assembly and repair. Equally down-regulated were transcripts involved in chlorophyll biosynthesis (e.g. *CHLOROPHYLL SYNTHASE*) and carbon fixation (e.g. *RUBISCO ACTIVASE* and *FRUCTOSE-1,6-BISPHOSPHATASE*) (Table A3.3 in Appendix III). Transcripts for proteins responsible of carbohydrate biosynthesis and transport (e.g. *SUCROSE PHOSPHATE SYNTHASE 4* and *SUGAR PHOSPHATE/PHOSPHATE TRANSLOCATOR*) were also generally down-expressed under LL, with few exceptions. Similarly to what observed for leaves, shared enriched biological processes in P-O SAMs under LL were associated to main phytohormones signaling pathways, namely *gibberellic acid mediated signaling pathway, regulation of jasmonic acid mediated signaling pathway, negative regulation of abscisic acid-activated signaling pathway* and *auxin efflux* (see Tables 3.10 and 3.11). In most cases, transcripts associated with these pathways were over-expressed, as for example many auxin carrier components.

Interestingly, many functions associated to the epigenetic regulation of gene expression were identified as enriched in SAMs under LL. In particular, GO-BP comprised e.g. *DNA methylation, histone H3-K9 methylation, nucleosome organization* and *chromatin silencing by small RNA* (Tables 3.10 and 3.11). DE transcripts included in these categories were generally down-regulated, and belonged to five main groups: histone proteins (e.g. *Histone H3.2, H4* and *H2A*), protein argonaute involved in RNA-mediated gene silencing (e.g. *ARGONAUTE 16*, 7 and 4A), DNA-binding factors involved in RNA-directed DNA methylation (RdDM) (e.g. *SAWADEE HOMEODOMAIN HOMOLOG 2*), transcriptional factors, and enzymes like histone methyltransferases, demethylase and acetyltransferase (e.g. *HISTONE H3-K9 METHYLTRANSFERASE 4*, *LYSINE-SPECIFIC DEMETHYLASE JMJ25*, *HISTONE DEACETYLASE* 19 and 14).

Shared up-regulated genes in LL included many transcripts encoding for antioxidants, chaperones, members of the protein ubiquitination pathway and general-stress proteins (e.g. *CHAPERONE PROTEIN DNAJ 8*, *E3 UBIQUITIN-PROTEIN LIGASE PUB23*, *1-CYS PEROXIREDOXIN, STRESS ENHANCED PROTEIN 2* and *UNIVERSAL STRESS PROTEIN A*). On the contrary, among shared down-regulated genes it is worth mentioning the protein *SLOWER GROWTH,* which is a component of the RNA exosome complex and

plays an important role in early seedling growth, and some RNA-binding proteins involved in leaf development and phloem/xylem histogenesis (e.g. *PENTATRICOPEPTIDE REPEAT-CONTAINING PROTEIN DOT4* and *VAN3-BINDING PROTEIN*). A fundamental light-responsive gene was also strongly down-regulated in the meristem of both shoot types, namely *LIGHT-DEPENDENT SHORT HYPOCOTYLS 3,* which is a developmental regulator and is required for SAM maintenance and formation of lateral organs. Other shared enriched BP were *positive regulation of transcription*, *response to sucrose*, *plant-type secondary cell wall biogenesis, positive gravitropism* and *response to far red light* (see Tables 3.10 and 3.11).

LL response of plagiotropic SAMs: In the meristem of plagiotropic shoots, fundamental responsive functions were enriched, as for example those related to plant development (*plant organ formation*, *cotyledon morphogenesis*, *cell wall modification involved in multidimensional cell growth, regulation of meristem growth*, *plant-type cell wall assembly*, *seed morphogenesis*) (see Fig. 3.16 and Table 3.10 for full list at FDR <0.01). Notably, many transcripts falling in abovementioned categories showed a reduced expressed in LL, such as *PROTEIN G1-LIKE4* (GIL4) that acts as a developmental regulator by promoting cell growth in response to light (logFC = -6.4).

BP categories related to gene transcription and signaling (e.g. *negative regulation of transcription DNA-templated*, *intracellular signal transduction),* DNA replication and repair, and cell cycle (e.g. *regulation of G2/M transition of mitotic cell cycle* and *double-strand break repair via homologous recombination*), were also among top GO enriched terms (see Fig. 3.16 and Table 3.10 for full list at FDR <0.01). Among DEGs included in these categories it is important to notice that many of them were down-regulated, including for example *BREAST CANCER SUSCEPTIBILITY 1 HOMOLOG*, which plays a major role in DNA repair and cell-cycle control/arrest, as well as transcripts encoding for cyclins (e.g. *CYCLIN-A1-1*) and other transcriptional factors (e.g. *CELL DIVISION CYCLE-ASSOCIATED 7*).

LL response of orthotropic SAMs: Under LL, a significant higher number of biological processes was enriched in the meristem of orthotropic shoots (see Fig. 3.17 and Table 3.11 for full list at FDR <0.01), in respect to plagiotropic ones. Top enriched functions included those related to chloroplast assembly and arrangement of constituent parts (*chloroplast organization, chloroplast RNA modification and chloroplast RNA processing*) that were not identified in plagiotropic shoots. A vast majority of transcripts involved in these processes were down-expressed in LL, for example the *PALE CRESS* protein, which is required for chloroplast differentiation, *RNA POLYMERASE SIGMA FACTOR SIGE* or *TOC75-3*, which

is an essential protein required for the import of protein precursors into chloroplasts. In addition, several enriched GO terms were also associated with plant development such as *leaf vascular tissue pattern formation* and *longitudinal axis specification.*

Other unique GO-BP were those related to sugar responses and signaling (e.g. *cellular response to sucrose starvation*, *sugar mediated signaling pathway* and *glucose metabolic process*) and amino acid metabolism (*regulation of cellular amino acid metabolic process* and *branched-chain amino acid catabolic process*). Enzymes with a role in sucrose starvation were generally over-expressed in LL (e.g. *2-OXOISOVALERATE DEHYDROGENASE SUBUNIT ALPHA 1*), as were genes involved in sugar mediated signaling pathway.

Stress-related biological functions were particularly represented in the contrast MO_LL-MO_C and included processed related to protein repair/degradation (*proteasomal ubiquitin-independent protein catabolic process* and *chaperone-mediated protein folding*), DNA damage responses (*DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest*) and apoptosis (*negative regulation of apoptotic process*). Curiously, many transcripts encoding for subunits of the proteasome complex were identified (e.g. *PAB1*, *PAF2*, *PBG1* etc.) and were all down-regulated under LL, whereas some proteins involved in DNA repair (e.g. *RAD5A*, *REV1* and *FACT COMPLEX SUBUNIT SSRP1)* were over-expressed.

DEGs involved in the blue light-signaling pathway had a mixed behavior; however, fundamental photoreceptors like *PHOTOTROPIN 1A* and cryptochromes were up-regulated in LL. The same was observed for some genes involved in long-day photoperiodism (e.g. *ZINC FINGER CONSTANS-LIKE 14*, *FT-INTERACTING PROTEIN 1*).

One enriched BP was particularly relevant, namely the *somatic stem cell population maintenance,* which contains both up and down-expressed transcripts.

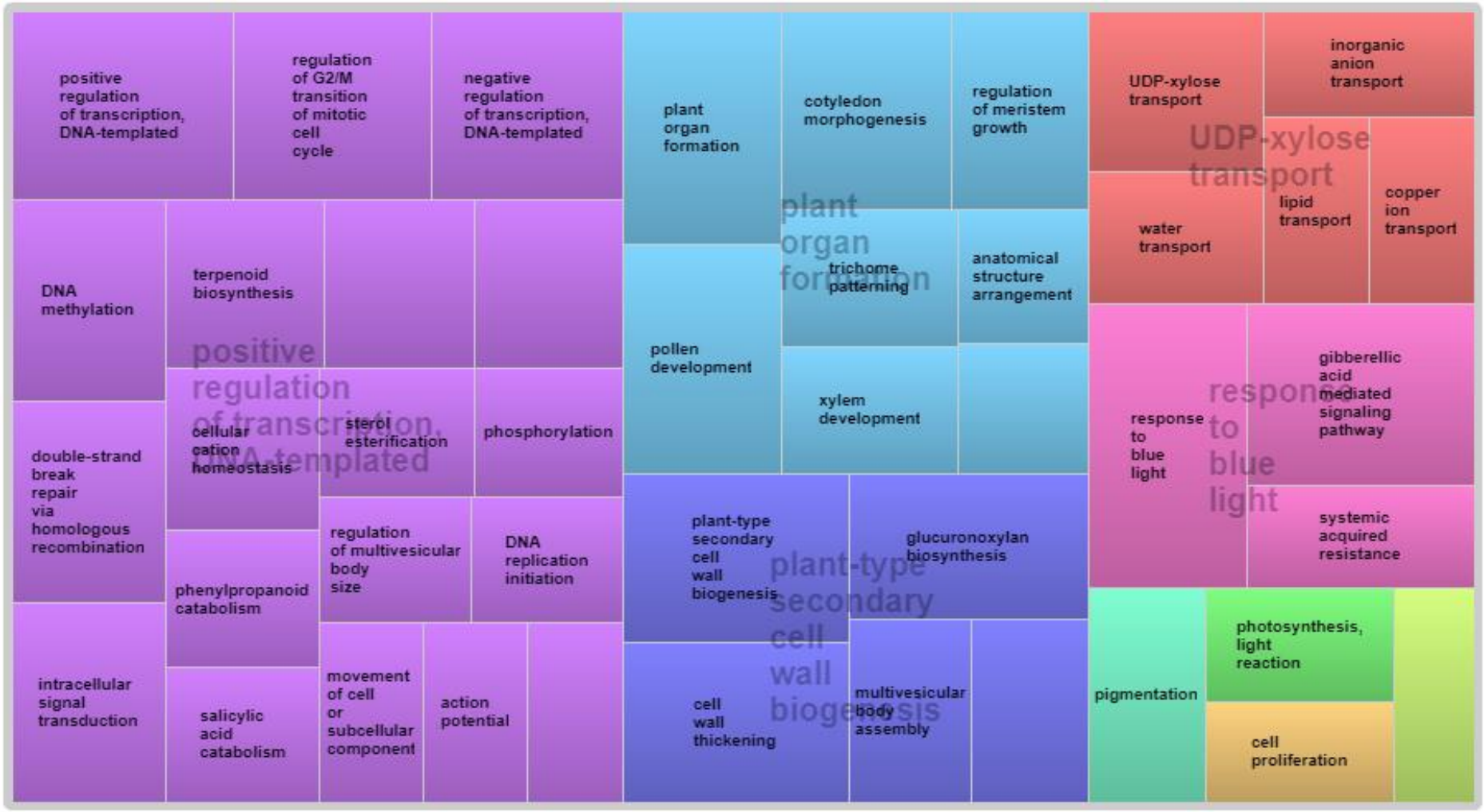

Fig. 3.16 TreeMap representation of GO enriched BP (FDR <0.01) in the contrast MP_LL-MP_C. GO terms are colored by semantic similarity and size reflects the abs_log10_pvalue.

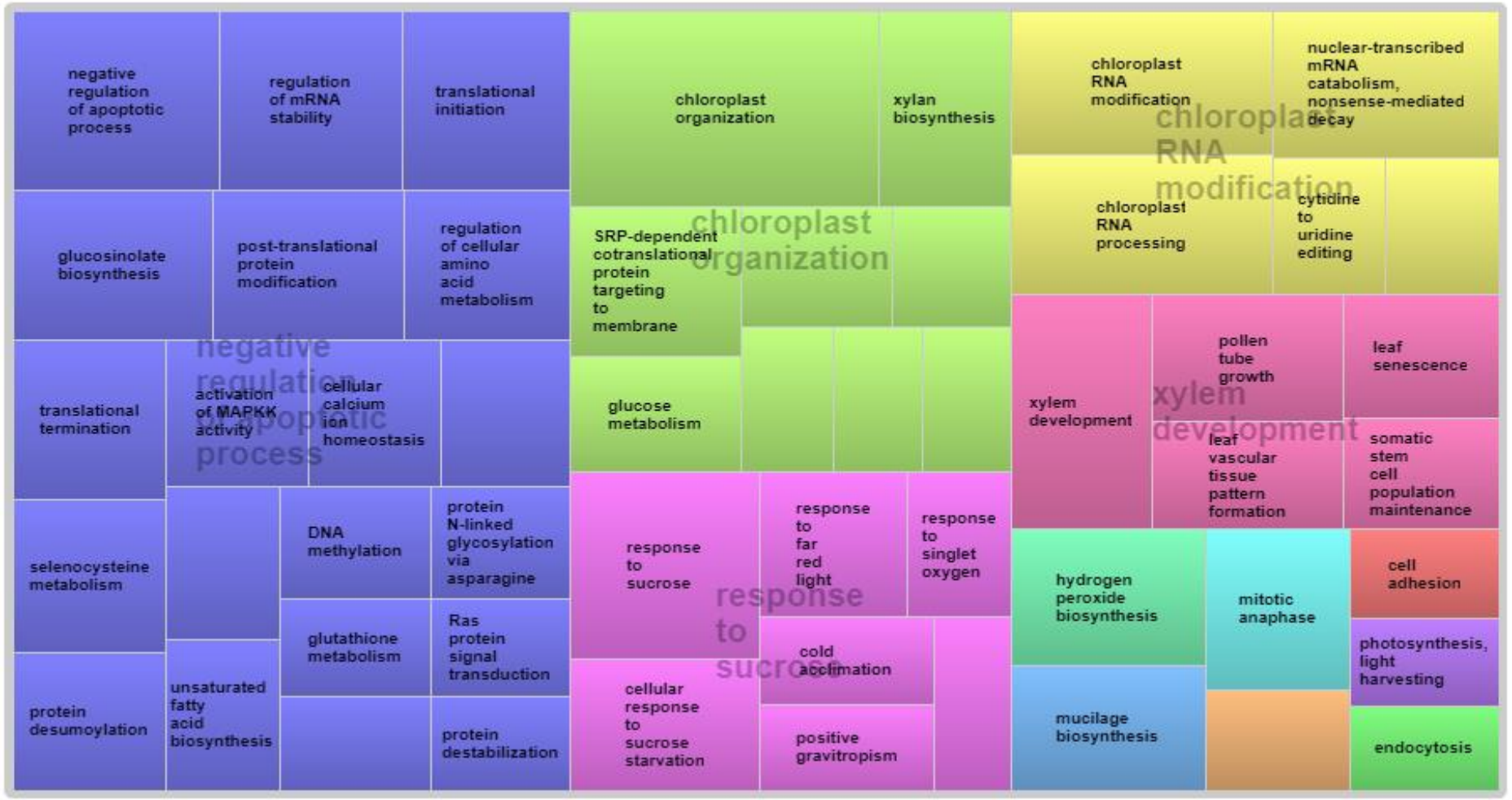

Fig. 3.17 TreeMap representation of GO enriched BP (FDR <0.01) in the contrast MO_LL-MO_C. GO terms are colored by semantic similarity and size reflects the abs_log10_pvalue.

Table 3.10 Reduced list of GO enriched BP (FDR <0.01) in MP_LL-MP_C. GO identifiers, BP names and FDR values are given. Shared BP are in bold.

GO ID	GO – Biological process	FDR	GO ID	GO – Biological process	FDR
0009637	response to blue light	1.08E-05	0009741	response to brassinosteroid	4.02E-03
0045893	**positive regulation of transcription, DNA-templated**	2.02E-05	0006098	pentose-phosphate shunt	4.10E-03
0009740	**gibberellic acid mediated signaling pathway**	2.66E-05	0009718	anthocyanin-containing compound biosynthetic process	4.42E-03
0009834	**plant-type secondary cell wall biogenesis**	6.41E-05	0030003	cellular cation homeostasis	4.43E-03
0010389	regulation of G2/M transition of mitotic cell cycle	9.28E-05	0043473	pigmentation	4.47E-03
1905393	plant organ formation	9.80E-05	0009788	**negative regulation of abscisic acid-activated signaling pathway**	5.07E-03
0009555	pollen development	1.17E-04	0010218	**response to far red light**	5.24E-03
0009958	**positive gravitropism**	1.28E-04	0048629	trichome patterning	5.61E-03
0052386	cell wall thickening	1.32E-04	0010315	**auxin efflux**	5.68E-03
0045892	negative regulation of transcription, DNA-templated	1.37E-04	0009269	response to desiccation	6.08E-03
0010417	glucuronoxylan biosynthetic process	1.99E-04	0009860	**pollen tube growth**	6.38E-03
0048826	cotyledon morphogenesis	2.86E-04	1901659	glycosyl compound biosynthetic process	7.09E-03
0007080	**mitotic metaphase plate congression**	2.95E-04	0055076	transition metal ion homeostasis	7.26E-03
0048354	mucilage biosynthetic process involved in seed coat development	5.00E-04	0009627	systemic acquired resistance	7.37E-03
0006306	**DNA methylation**	6.62E-04	0071668	plant-type cell wall assembly	7.43E-03
0000724	double-strand break repair via homologous recombination	6.64E-04	0000911	cytokinesis by cell plate formation	7.47E-03
0035556	intracellular signal transduction	7.72E-04	0009411	response to UV	7.48E-03
2000022	**regulation of jasmonic acid mediated signaling pathway**	1.20E-03	0006833	water transport	7.90E-03
0042547	cell wall modification involved in multidimensional cell growth	1.28E-03	0051555	flavonol biosynthetic process	8.14E-03
0015790	UDP-xylose transmembrane transport	1.80E-03	0048317	seed morphogenesis	8.80E-03
0009749	response to glucose	2.00E-03	0015698	inorganic anion transport	9.36E-03
0030243	cellulose metabolic process	2.25E-03	0009744	**response to sucrose**	9.61E-03
0010075	regulation of meristem growth	2.25E-03	0010089	**xylem development**	9.80E-03
0016114	terpenoid biosynthetic process	2.76E-03	0036258	multivesicular body assembly	9.80E-03
0090307	**mitotic spindle assembly**	2.84E-03			

Table 3.11 Reduced list of GO enriched BP (FDR <0.01) in MO_LL-MO_C. GO identifiers, BP names and FDR values are given. Shared BP are in bold.

GO ID	GO – Biological process	FDR	GO ID	GO – Biological process	FDR
0045893	**positive regulation of transcription, DNA-templated**	3.16E-14	0034728	nucleosome organization	1.76E-03
0009658	chloroplast organization	2.38E-10	0010315	**auxin efflux**	1.80E-03
1900865	chloroplast RNA modification	1.00E-06	0035019	somatic stem cell population maintenance	1.80E-03
0043066	negative regulation of apoptotic process	1.23E-06	0006306	**DNA methylation**	2.01E-03
0031425	chloroplast RNA processing	1.30E-06	0044092	negative regulation of molecular function	2.38E-03
0009744	**response to sucrose**	2.16E-06	0010017	red or far-red light signaling pathway	2.60E-03
0000184	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	4.47E-06	0009942	longitudinal axis specification	2.67E-03
0010089	**xylem development**	5.50E-06	0010540	basipetal auxin transport	2.90E-03
0043488	regulation of mRNA stability	6.02E-06	0007281	germ cell development	2.96E-03
0010499	proteasomal ubiquitin-independent protein catabolic process	1.15E-05	0018279	protein N-linked glycosylation via asparagine	3.03E-03
0006413	translational initiation	1.58E-05	0007089	traversing start control point of mitotic cell cycle	3.25E-03
0019761	glucosinolate biosynthetic process	1.59E-05	0016554	cytidine to uridine editing	3.32E-03
2000031	regulation of salicylic acid mediated signaling pathway	1.92E-05	0009083	branched-chain amino acid catabolic process	3.37E-03
0009788	**negative regulation of abscisic acid-activated signaling pathway**	2.64E-05	0080001	mucilage extrusion from seed coat	3.59E-03
0043687	post-translational protein modification	2.65E-05	0009631	cold acclimation	3.83E-03
0050665	hydrogen peroxide biosynthetic process	6.21E-05	0000304	response to singlet oxygen	3.84E-03
0045492	xylan biosynthetic process	7.41E-05	0009958	**positive gravitropism**	4.04E-03
0006614	SRP-dependent cotranslational protein targeting to membrane	7.81E-05	0090307	**mitotic spindle assembly**	4.46E-03
0080156	mitochondrial mRNA modification	9.01E-05	0048767	root hair elongation	4.50E-03
0043617	cellular response to sucrose starvation	1.01E-04	0006749	glutathione metabolic process	4.57E-03
0006521	regulation of cellular amino acid metabolic process	1.02E-04	0061077	chaperone-mediated protein folding	4.60E-03
0006415	translational termination	1.22E-04	0010114	response to red light	4.62E-03
0009860	**pollen tube growth**	1.24E-04	0007080	**mitotic metaphase plate congression**	4.83E-03
0010192	mucilage biosynthetic process	1.29E-04	0010889	regulation of sequestering of triglyceride	5.00E-03
0016259	selenocysteine metabolic process	1.87E-04	0048653	anther development	5.19E-03

2000022	**regulation of jasmonic acid mediated signaling pathway**	1.90E-04	0031048	chromatin silencing by small RNA	5.29E-03
0000090	mitotic anaphase	2.08E-04	0070370	cellular heat acclimation	5.41E-03
0009750	response to fructose	2.99E-04	0010264	myo-inositol hexakisphosphate biosynthetic process	5.41E-03
0006595	polyamine metabolic process	3.13E-04	0006650	glycerophospholipid metabolic process	5.57E-03
0030244	cellulose biosynthetic process	3.62E-04	0010413	glucuronoxylan metabolic process	6.22E-03
0010218	**response to far red light**	3.94E-04	0007265	Ras protein signal transduction	6.30E-03
0016926	protein desumoylation	4.22E-04	0010252	auxin homeostasis	6.32E-03
0000186	activation of MAPKK activity	4.40E-04	0008284	positive regulation of cell proliferation	6.46E-03
0010305	leaf vascular tissue pattern formation	4.85E-04	0010082	regulation of root meristem growth	6.69E-03
0006627	protein processing involved in protein targeting to mitochondrion	5.59E-04	0051775	response to redox state	6.98E-03
0009834	**plant-type secondary cell wall biogenesis**	5.70E-04	1903086	negative regulation of sinapate ester biosynthetic process	6.98E-03
0010182	sugar mediated signaling pathway	5.95E-04	0007155	cell adhesion	7.36E-03
0006006	glucose metabolic process	6.26E-04	0006997	nucleus organization	7.49E-03
0009825	multidimensional cell growth	6.33E-04	0048834	specification of petal number	7.56E-03
0051098	regulation of binding	7.14E-04	0048574	long-day photoperiodism, flowering	7.96E-03
0002223	stimulatory C-type lectin receptor signaling pathway	7.55E-04	0019395	fatty acid oxidation	8.10E-03
0006874	cellular calcium ion homeostasis	7.87E-04	0009251	glucan catabolic process	8.19E-03
0033209	tumor necrosis factor-mediated signaling pathway	8.20E-04	0006835	dicarboxylic acid transport	8.28E-03
0048359	mucilage metabolic process involved in seed coat development	8.60E-04	0031648	protein destabilization	8.32E-03
0010150	leaf senescence	8.65E-04	0098662	inorganic cation transmembrane transport	8.44E-03
0048010	vascular endothelial growth factor receptor signaling pathway	1.01E-03	0007143	female meiotic nuclear division	8.49E-03
0006977	DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest	1.01E-03	0080086	stamen filament development	8.54E-03
0043928	exonucleolytic nuclear-transcribed mRNA catabolic process involved in deadenylation-dependent decay	1.02E-03	0009765	photosynthesis, light harvesting	8.56E-03
0000084	mitotic S phase	1.03E-03	0006897	endocytosis	9.36E-03
0009827	plant-type cell wall modification	1.03E-03	0031124	mRNA 3'-end processing	9.45E-03
0000122	negative regulation of transcription by RNA polymerase II	1.23E-03	0043489	RNA stabilization	9.55E-03
0009640	photomorphogenesis	1.26E-03	0033108	mitochondrial respiratory chain complex assembly	9.57E-03

0052544	defense response by callose deposition in cell wall	1.28E-03	0008334	histone mRNA metabolic process	9.68E-03
0006470	protein dephosphorylation	1.45E-03	0006260	DNA replication	9.87E-03
0030422	production of siRNA involved in RNA interference	1.52E-03	0007076	mitotic chromosome condensation	1.00E-02
0009740	**gibberellic acid mediated signaling pathway**	1.55E-03	0033962	cytoplasmic mRNA processing body assembly	1.00E-02
0009785	blue light signaling pathway	1.68E-03			
0006636	unsaturated fatty acid biosynthetic process	1.72E-03			

3.4 Discussion

Low-light stress represents one of the most pressing threat affecting seagrass performance and survival, due to direct and indirect human-related factors (e.g. excess anthropogenic nutrients leading to eutrophication, increased sediment accretion and resuspension, aquaculture and dredging, as well as regional weather patterns) (Ralph et al. 2007).

The experiment presented in this chapter aimed to assess the response to low-light stress in *P. oceanica*, at different hierarchical levels. From one side, I investigated putative differences in the response of plagiotropic *vs.* orthotropic shoots, while from the other side I explored the differential behavior of different organs, namely the shoot-apical meristem and leaves. Notably, the existence of differences in the response of distinct shoot types and of different organs has been neglected in previous research on the effects of light limitation (and other abiotic stressors) in seagrasses.

For the first objective of the study, I wanted to get more insights into the biological and ecological role of the different shoot types present within *P. oceanica* clones. The underlying hypothesis was that, under stress, metabolic rearrangements occurring in vertical shoots would be devoted to provide resources for apical ones, representing the leading plant of the clone, to withstand the unfavorable event (Liu et al. 2016). In this context, the analysis of the plasticity in the response of vertical and apical shoots, by means of whole transcriptome sequencing, was used to find molecular signatures of clonal integration. So far, a differential transcriptome analysis of apical and vertical shoots has never been conducted in seagrasses, yet apical shoots are typically avoided in stress-related studies, for their recognized peculiar physiological behavior, which could provide misleading results. On the other hand, accurate analyses of their metabolism, primarily at molecular level, have hitherto never be performed.

The second object of the study was motivated by the current search for early warning indicators of stress in seagrasses, with the goal of detecting stress before plants "pass the point of no return" (Macreadie et al. 2014; Traboni et al. 2018). Although it is increasingly recognized that "classical" morphological and physiological monitoring methods do not always provide a sufficient timeframe for successful remedial actions to take place (Ceccherelli et al. 2018), the use of molecular tools that would change at the onset of stress, have rarely been applied in seagrasses (Pernice et al. 2015). One question that is apparently undervalued is where to look for such indicators; does the leaf really represent a good proxy of imminent shoot mortality? With this in mind, the examination of the transcriptomic response of the shoot-apical meristem was performed and compared with that of leaf tissues.

In the following sections, I discuss key findings of this study, with a special focus on questions and hypothesis discussed above.

3.4.1 *Main molecular, physiological and morphological responses to LL*

Photo-physiological responses in terms of changes in photosynthetic parameters and pigment content were evaluated after 15 (T1) and 30 (T2) days of exposure to 80 % light reduction. Light limitation had no effects on dark-adapted chlorophyll *a* fluorescence derived parameters (F_0 and Fv/Fm), whereas it strongly affected some RLC-derived parameters. Specifically, a large reduction (about 30-40%) in the effective quantum yield of PSII (ΔF/Fm'), electron transport rate (r-ETR), and minimum saturating irradiance (Ik) was observed in light-limited plants, without any differences between plagiotropic and orthotropic shoots. No differences in NPQ and pigment content were recorded along the experiment, although there was a tendency for pigments to decrease in LL, especially in plagiotropic shoots at T2. In LL, seagrass photosynthetic performance is generally enhanced through a range of photo-acclimative responses leading to an increase in light utilization efficiency and reduced respiratory rates of leaf tissue, resulting in lower minimum light requirement for photosynthesis (Ralph et al. 2007). Fluorescence-based estimates of light limitation in seagrasses have generally demonstrated an increase in photosynthetic efficiency with LL, a reduced electron transport rate and saturating irradiance (see Ralph et al. 2007 and reference therein). These photo-physiological acclimation strategies are typical of shade-adapted plants (Kirk 2010).

Findings presented here are consistent with these observations, and with previous reports for *P. oceanica* (Dattolo et al. 2014; Dattolo et al. 2017; Procaccini et al. 2017). Dattolo et al. (2017) reported photo-physiological measurements for deep and shallow *P. oceanica* plants exposed to natural and reciprocal light regimes in mesocosms. In this study, Fv/Fm showed no significant modification as a consequence of the light treatment, whereas ΔF/Fm' and rETR displayed significantly lower values in deep than in shallow plants before the light change, then the effects were reverted when exposed to reciprocal light levels.

Shade acclimation generally involves the enhancement of light harvesting capacity, typically achieved through a higher pigment content. This was not the case for *P. oceanica*, as no difference in pigment concentration were recorded along the experiment, with generally slightly higher values in control light plants, in respect to LL ones. However, pigment concentration does not seem to vary coherently to light availability in *P. oceanica*. Higher pigment concentration has been generally found for high light (shallow) plants rather than

deep-growing ones (Dattolo et al. 2014; Dattolo et al. 2017), or no differences have been detected in other cases (e.g. Procaccini et al. 2017).

At morphological level, there was a tendency for plagiotropic and orthotropic shoots under LL, to reduce the number of leaves and their maximum length. An overall reduction in shoot size was especially evident at T2 and T3, for both shoot types. At meadow scale, *P. oceanica* acclimation to LL (e.g. with increasing depth) involves the progressive reduction of the canopy complexity (e.g. shoot density and canopy height), in order to regulate the available light (Dalla Via et al. 1998; McMahon et al. 2013; Marín-Guirao et al. 2015). These structural changes are considered the main adaptive mechanisms to offset depth-related light reductions, especially in large-sized seagrasses (Olesen et al. 2002; Ralph et al. 2007; Collier et al. 2008). Therefore, results presented here are in line with a general strategy to maximize light exposure of photosynthetic tissues and minimize shoot respiratory demands in LL (Ralph et al. 2007; Dattolo et al. 2017). LL exposed plants also showed a great reduction in leaf growth rate at all sampling time points, with the largest reduction (78%) observed in apical shoots at T3.

At molecular level, the reduction of photosynthetic capacity under LL reflected the down-expression of many transcripts encoding for photosynthesis-related components, and enzymes involved in chlorophyll biosynthesis and carbon assimilation pathways (Table A3.3). This has been frequently observed in leaves of *P. oceanica* (Dattolo et al. 2014; Procaccini et al. 2017) or other seagrass species such as *Z. muelleri* (Davey et al. 2018) under light limitation. Surprisingly, the number of DEGs associated to abovementioned processes was actually much higher in the analysis of plagiotropic and orthotropic SAMs, rather than leaves. Accordingly, the GO term *photosynthesis, light reaction* was found enriched in the SAM transcriptome of both shoot types, and not in leaves. Down-regulated transcripts included constituents of PSI and PSII and their antenna complexes, proteins assisting photosystem assembly (e.g. *LOW PSII ACCUMULATION 3*) and members of the photosynthetic electron transport chain. Similarly, LL slowed down the accumulation of key transcripts involved in chlorophyll biosynthesis and Calvin cycle, such as the RuBisCO activator *RUBISCO ACTIVASE* 1-2. In addition, the *SIGE* factor, which recruits plastid-encoded RNA polymerase to specific initiation sites (e.g. *psbA* and *psbD*) and initiate gene transcription (Chi et al. 2015) was also among down-expressed genes.

These results demonstrate that the expression of constituents of the machineries that drive the light-dependent and -independent reactions of photosynthesis, which correlates directly with chloroplast development, starts already in the SAM of *P. oceanica*. In further support of this, it is worth mentioning that, at least in the meristem of orthotropic shoots, the GO

terms *chloroplast organization*, *chloroplast RNA modification* and *chloroplast RNA processing* were found among top-enriched ones. This was not an obvious observation, as for instance in maize (a monocot), very few photosynthetic-related genes are found to be expressed in the SAM and leaf primordia (Brooks III et al. 2009). On the contrary, results presented here are quite similar to those found for the shoot apex of tomato (a dicot), where the presence of transcripts for different chloroplast functions was already detected in the stem cell-containing region of the SAM, revealing an early acquisition of photosynthetic capacity (Dalal et al. 2018).

The second important consideration is that the vast majority of these DEGs were down-regulated in plants exposed to limiting light, suggesting that LL stress could significantly impair the SAM transcriptional machinery that operate for chloroplast biogenesis and later for the establishment of photosynthetic competence. RNA-seq analysis was performed after only 15 days of exposure; this timeframe apparently did not largely compromise the expression of photosynthesis-related transcripts in the leaves, rather SAM-related changes in gene expression anticipate leaf responses.

In addition to genes related to photosynthesis, some transcripts encoding for proteins responsible of carbohydrate biosynthesis (e.g. *SUCROSE PHOSPHATE SYNTHASE 4*) and transport were also generally down-expressed under LL, in both leaves and SAMs. Sucrose phosphate synthase catalyzes the rate-limiting step of sucrose biosynthesis from UDP-glucose and fructose- 6-phosphate, thus playing a major role for plant sucrose availability. Although here a direct estimation of plant sugar content was not performed, a reduced carbohydrate content in leaves and rhizomes under LL has been previously demonstrated for *P. oceanica* in both mesocosm (Dattolo et al. 2017) and field experiments (Ruiz and Romero 2001). As much as 80% of the CO_2 assimilated during photosynthesis is channeled into synthesis of sucrose, which is the major organic carbon form exported from source to sink organs (Rosa et al. 2009). LL reduction of photosynthesis and consequent sucrose synthesis, could have contribute to the depletion of carbohydrate reserves from storage organs (e.g. rhizomes), leading to the observed negative impacts on *P. oceanica* growth and survival in the medium term.

3.4.2 *Epigenetic mechanisms involved in the LL response*

Interestingly, many functions associated with the epigenetic regulation of gene expression were identified as enriched in plants under LL, especially in the analysis of SAMs, where the GO terms *DNA methylation* and *chromatin silencing by small RNA* were found among

top enriched BP (FDR <0.01). Other functions related to histone modifications and small RNA-based epigenetic changes were enriched in SAMs at lower significance level (FDR <0.05) (data not shown) and include *histone H3-K9 methylation*, *histone H3-K36 dimethylation*/*trimethylation* and *regulation of histone acetylation*. Among DEGs included in these categories, those involved in DNA methylation (e.g. *WD repeat-containing protein*), transcripts for histone proteins (*H3.2*, *H4*, *H2A.6* and *H2A variant 3*), protein argonaute (*16*, 7 and 4A) and DNA-binding factors involved in RdDM, were generally down-regulated in LL. Enzymes involved in histone modifications showed a variable behavior; deacetylases were all up-regulated, whereas methyltransferases were either up or down-regulated. As discussed also in previous chapters, epigenetic mechanisms associated with DE transcripts listed above (DNA methylation, histone modifications, placement of histone variants and regulation by noncoding RNA) play an essential role in modulating chromatin structure and function and subsequent gene activity, and are associated to both developmental processes and stress response (Mirouze and Paszkowski 2011; Gutzat and Mittelsten Scheid 2012). The significance of these epigenetic marks differs according to the location of the modified sites (on DNA or proteins), and on the type of chemical modification (Liu et al. 2010; Niederhuth and Schmitz 2017). For example, the deacetylation of lysine residues of histones by histone deacetylase links to transcriptional repression and gene silencing (Luo et al. 2017), as for histone H3-K9 methylation; on the contrary, H3-K36 methylation is generally associated with active genes (Liu et al. 2010).

Another important case is that of protein Argonaute (Ago). Ago proteins are ubiquitously expressed and bind to siRNAs or miRNAs to guide post-transcriptional RNA-induced gene silencing, either by destabilization of the mRNA or by translational repression (Peters and Meister 2007; Höck and Meister 2008). They play important roles in plant growth, development and stress response (Xu et al. 2016). Some Ago proteins, such as *AGO7* that was found down-regulated in the SAM of apical shoots, are directly implicated in the regulation of shoot apical meristem initiation and maintenance (Nagasaki et al. 2007; Zhang and Zhang 2012). Specifically, mutation in *AGO7* and other components of small interfering RNA production pathway, can cause complete deletion or abnormal formation of the SAM in rise (Nagasaki et al. 2007). Other Ago proteins, as for instance *AGO4*, participate in epigenetic DNA modifications through the RdDM pathway. *AGO4* mutant phenotype in *Arabidopsis* is associated with loss of epigenetic modifications at many chromosomal loci, including transposons (Zilberman et al. 2003; Qi et al. 2006; Havecker et al. 2010).

In addition to transcripts involved in the epigenetic regulation of gene expression, many DEGs under LL were associated to retroelements, specifically to retrotransposons of the

Copia family and retroviral-like transposon Tnt 1-94, in both leaves and SAMs. Notably, epigenetic mechanisms are intimately linked to the activity of transposable elements, since alternative epigenetic states can promote or prevent the movement of DNA transposons and retroelements (Mirouze and Paszkowski 2011).

These results highlight the importance of epigenetic mechanisms in activating the short-term response to low-light stress in *P. oceanica*, as it was previously demonstrated for heat stress (Marín-Guirao et al. 2017). Importantly, besides having a fundamental role in the immediate acclimation response to abiotic stressors, epigenetic mechanisms could have a role also in the long-term plant stress adaptation, due to the heritability of epi-alleles and regulation of transposon mobility (Mirouze and Paszkowski 2011).

3.4.3 *Molecular signatures of clonal integration under LL*

Interestingly, although no substantial differences were observed in the photo-physiological response of plagiotropic *vs.* orthotropic shoots in LL, with the exception of some variability in the leaf growth rate, the whole transcriptome analysis revealed different metabolic processes enriched in the two shoot types, and only a small portion of shared DEGs. Another important consideration is that, although a higher number of DEGs was generally found for apical shoots, in respect to vertical ones, a lower number of enriched biological processes was associated with the former, in both leaves and SAMs. This suggests that, under LL, the response of apical shoots is less complex and restricted to few important functions, whereas that of vertical shoots is more heterogeneous and involved a wide variety of processes.

Among the few enriched BP in leaves of plagiotropic shoots under limiting light, top GO terms were those associated with the regulation of phytohormone signaling pathways and response to hormones (e.g. *negative regulation of cytokinin-activated signaling pathway* and *response to abscisic acid*), and many transcripts within these categories were found as up-regulated in LL. In addition, several genes involved in the auxin-activated signaling pathway and transport, were also among top over-expressed genes. One intriguing hypotheses behind these observations is that *P. oceanica* plants might use hormone signaling to modify patterns of resource sharing between ramets under light shortage. It has been demonstrated that carbon and/or nitrogen translocation occurs among seagrass ramets, and resource translocation tends to proceed, in most cases, towards the rhizome apices, which represent the expanding edges of the clones (Harrison 1978; Terrados et al. 1997b; Marbà et al. 2002; Marbà et al. 2006). Importantly, this asymmetrical resource mobilization inside the clone plays an important role in supporting seagrass clonal growth (Marbà et al. 2002). In

terrestrial systems, there is considerable evidence that hormones can cause differences between biomass of plant parts in response to different resource availability, and also regulate translocation between branches within shoots (Voesenek and Blom 1996). In particular, two major types of plant hormone, auxin and cytokinin, can direct the transport of carbohydrates and nutrients between different plant parts (Morris and Arthur 1987; Cole and Patrick 1998; Javid et al. 2011). For example, in the terrestrial clonal plant *Fregaria chiloensis*, it has been demonstrated that ramets treated with auxin showed greater carbon (C) and nitrogen (N) import from connected ramets, especially when those were located in resource poor microsites (e.g. light poor or N-poor) (Alpert et al. 2002).

Findings presented here might indicate that hormones (e.g. auxins or cytokinins), could modify patterns of resource sharing between ramets in seagrasses and eventually enhance resource concentration in particular ramets of the clone, as apical shoots.

Although this experiment cannot provide direct evidence supporting this hypothesis, it is worth mentioning that the other few GO terms enriched in P_LL-P_C were associated to secondary metabolite-related metabolic processes (e.g. *regulation of phenylpropanoid metabolism* and *flavonoid metabolic process*). Flavonoids, in particular, a subgroup of phenylpropanoid compounds whose synthesis is dependent upon environmental conditions (e.g. light and temperature), represent a hallmark of stressed plants. In addition, they seem to be negative regulators of polar auxin transport, thus enhancing localized auxin accumulation, and activating auxin-dependent stress responses (Peer and Murphy 2007; Bielach et al. 2017). By controlling the processes of phytohormone transport and distribution, flavonoids could indirectly modulate patterns of resource accumulation in *P. oceanica* ramets under low light stress (Peer and Murphy 2007; Bielach et al. 2017).

3.4.4 *Response of the shoot-apical meristem to LL: a new early warning indicator?*

Under limiting light, the number of DEGs and GO enriched biological processes identified in plagiotropic and orthotropic SAMs was always greater than that identified for leaves. As previously discussed, a large portion of DEGs associated to photosynthesis, carbon assimilation and carbohydrate biosynthesis, was found in the SAM analyses. Other enriched functions included fundamental processes related to light sensing, meristem growth and maintenance, cell proliferation, development of plant organs, as well as DNA damage/repair mechanisms. Overall, the over-expression of transcripts related to CSR mechanisms and light perception/signaling was observed in LL, in correspondence with a down-regulation of functions related to cell proliferation and organ/tissue development.

The negative regulation of functions related to cell growth and proliferation is particularly relevant, since this is one of the key response of plants to non-lethal abiotic/biotic stressors (Kitsios and Doonan 2011). As discussed in previous sections, LL slowed-down overall shoot size and leaf growth rate in *P. oceanica*. This reduction in plant size can be attributed to a reduction in cell number, as well as cell growth that starts primarily at meristem level (Kitsios and Doonan 2011). Cell enlargement is modulated in response to stress by the plant growth hormone gibberellin (Razem et al. 2006); notably, the BP *gibberellic acid mediated signaling pathway* was among top enriched GO terms in MP and MO under LL. In addition, a reduction in cell number can be hypothesized and attributed to the observed suppression of cell cycle-related transcripts (e.g. cyclins), resulting in cell-cycle arrest at the G1/S and G2/M checkpoints, prolonged S-phase progression and/or delayed entry into mitosis (De Veylder et al. 2007). The inactivation of genes required for cell-cycle progression can arise from the activation of DNA stress checkpoints, which induces also DNA-repair related genes. This coordinated action ensures that cells repair their damaged genome before they proceed into mitosis (De Veylder et al. 2007). SAM analyses under LL stress revealed the presence of many GO terms associated with DNA damage (e.g. *DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest* and *double-strand break repair via homologous recombination*). Some genes related to those processes were actually found over-expressed in LL (e.g. *RAD5A* and *REV1*) however, DNA-repair associated transcripts were also found among down-regulated genes (e.g. *BRCA1*), suggesting that inhibition of cell proliferation and cell-cycle arrest could be associated also to other processes, most likely sucrose starvation (Yu 1999; Riou-Khamlichi et al. 2000). Lastly, LL stress strongly affected developmental processes related to plant organ/tissue formation, as for instance phloem/xylem histogenesis. Several transcripts related to those functions were suppressed in LL, as the important developmental regulator *LIGHT-DEPENDENT SHORT HYPOCOTYLS 3*, which is required for SAM maintenance and formation of lateral organs (Cho and Zambryski 2011).

In conclusion, this experiment revealed that the stress response in *P. oceanica* exposed to light limitation starts primarily at the level of meristems, which are the most sensitive plant parts, with the lowest tolerance threshold. Meristem response to LL was much more complex and likely anticipated leaf-related response. This is reflected primarily by the strong down-regulation of genes related to photosynthesis, carbon assimilation, carbohydrate biosynthesis and cell growth/proliferation occurring in the SAM. These molecular responses are directly implicated in the physiological and morphological responses observed at leaf and whole-plant levels. Moreover, this research sheds first light on the role of plagiotropic *vs.*

orthotropic shoots and proposes some mechanisms that could underline clonal integration mechanisms in response to stress. Although further investigations are certainly needed, it seems that orthotropic shoots “do most of the job”, whereas enriched functions of apical shoots were restricted to few important processes, such as hormone-related signaling pathways. These transcriptome data offer great opportunity for future exploration of important mechanisms, as for instance plant hormone signal transduction and p53 signaling pathways, as well as those involved in regulating the pluripotency of stem cells (Figs. A3.3, A3.4 and A3.5), which are completely unknown in seagrasses. In addition, SAM-related gene expression response could be taken as a fundamental indicator of seagrass status under stress.

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Appendix III

Table A3.1 Number of row-sequencing reads and final number of cleaned reads after quality controls.

Name	Treatment	Shoot type	Raw reads	Cleaned reads
TLA1L_S1_R1_001.fastq	Control	Plagiotropic	21,713,061	18,480,765
TLA2L_S2_R1_001.fastq	Control	Plagiotropic	24,404,020	20,792,520
TLA3L_S3_R1_001.fastq	Control	Plagiotropic	19,112,019	15,754,602
TLA4L_S4_R1_001.fastq	LL	Plagiotropic	21,047,258	17,977,024
TLA5L_S5_R1_001.fastq	LL	Plagiotropic	17,561,593	14,974,037
TLA6L_S6_R1_001.fastq	LL	Plagiotropic	21,756,207	18,413,611
TLV1L_S13_R1_001.fastq	Control	Orthotropic	24,233,448	20,600,808
TLV2L_S14_R1_001.fastq	Control	Orthotropic	20,972,421	17,781,971
TLV3L_S15_R1_001.fastq	Control	Orthotropic	25,207,915	21,396,476
TLV4L_S16_R1_001.fastq	LL	Orthotropic	18,750,839	15,776,205
TLV5L_S17_R1_001.fastq	LL	Orthotropic	19,252,684	16,144,524
TLV6L_S18_R1_001.fastq	LL	Orthotropic	14,649,215	12,413,721
		Total_leaves	**248,660,680**	**210,506,264 (84.66%)**
TLA1S_S7_R1_001.fastq	Control	Plagiotropic	21,217,720	18,001,701
TLA2S_S8_R1_001.fastq	Control	Plagiotropic	19,324,896	16,401,032
TLA3S_S9_R1_001.fastq	Control	Plagiotropic	20,613,466	17,509,224
TLA4S_S10_R1_001.fastq	LL	Plagiotropic	21,854,562	18,559,501
TLA5S_S11_R1_001.fastq	LL	Plagiotropic	18,558,792	15,415,138
TLA6S_S12_R1_001.fastq	LL	Plagiotropic	21,270,253	18,124,773
TLV1S_S19_R1_001.fastq	Control	Orthotropic	15,113,973	12,712,926
TLV2S_S20_R1_001.fastq	Control	Orthotropic	24,367,928	20,615,715
TLV3S_S21_R1_001.fastq	Control	Orthotropic	22,314,800	18,838,891
TLV4S_S22_R1_001.fastq	LL	Orthotropic	22,511,592	19,100,089
TLV5S_S23_R1_001.fastq	LL	Orthotropic	22,508,855	19,064,032
TLV6S_S24_R1_001.fastq	LL	Orthotropic	19,699,597	16,638,185
		Total_SAMs	**249,356,434**	**210,981,207 (84.61%)**
		TOTAL	**498,017,114**	**421,487,471 (84.63%)**

Table A3.2 Photosynthetic parameters and pigment concentrations determined at T1 and T2 in plagiotropic and orthotropic *P. oceanica* shoots. r-ETR (µmol electrons $m^{-2} s^{-1}$); Ik (µmol photons $m^{-2} s^{-1}$); Chl *a*, *b* and carotenoids (µg cm^{-1}); Chl *b/a* (molar ratio). P_C = plagiotropic shoots, control conditions; O_C = orthotropic shoots, control conditions; P_LL = plagiotropic shoots, low-light conditions; O_LL = orthotropic shoots, low-light conditions. Values are means (SE) for *n*=3.

	F_0	Fv/Fm	ΔF/Fm'	r-ETR	Ik	NPQ	Chl*a*	Chl*b*	Carotenoids	Chl *b/a*
T1										
P_C	266.000 (10.69)	0.793 (0.00)	0.355 (0.03)	42.064 (5.21)	103.421 (16.39)	0.473 (0.02)	36.207 (4.78)	16.143 (2.17)	9.849 (1.21)	0.439 (0.01)
O_C	262.167 (2.24)	0.789 (0.00)	0.358 (0.06)	41.761 (7.66)	105.351 (14.74)	0.425 (0.05)	35.344 (1.89)	15.846 (0.54)	9.500 (0.82)	0.443 (0.01)
P_LL	248.667 (7.42)	0.796 (0.00)	0.268 (0.03)	28.378 (2.78)	60.728 (4.50)	0.521 (0.07)	36.123 (3.04)	15.900 (1.20)	9.745 (0.88)	0.434 (0.01)
O_LL	258.667 (5.73)	0.793 (0.00)	0.231 (0.01)	24.337 (1.20)	55.026 (3.19)	0.435 (0.03)	37.019 (4.60)	16.527 (2.00)	10.670 (1.65)	0.440 (0.01)
T2										
P_C	249.833 (5.92)	0.792 (0.00)	0.456 (0.03)	49.534 (3.36)	112.157 (6.18)	0.464 (0.04)	38.139 (6.49)	18.034 (2.18)	10.179 (1.78)	0.476 (0.04)
O_C	257.333 (10.10)	0.794 (0.00)	0.412 (0.04)	45.261 (4.37)	103.894 (8.95)	0.529 (0.06)	30.541 (2.12)	13.873 (1.02)	8.462 (0.53)	0.447 (0.01)
P_LL	239.000 (7.01)	0.798 (0.00)	0.285 (0.03)	28.743 (2.15)	66.561 (3.45)	0.516 (0.04)	30.487 (6.31)	12.788 (2.80)	8.178 (1.72)	0.410 (0.01)
O_LL	238.167 (9.44)	0.797 (0.00)	0.244 (0.02)	26.505 (2.58)	66.135 (2.81)	0.482 (0.07)	30.911 (4.15)	14.971 (2.47)	6.753 (0.84)	0.475 (0.03)

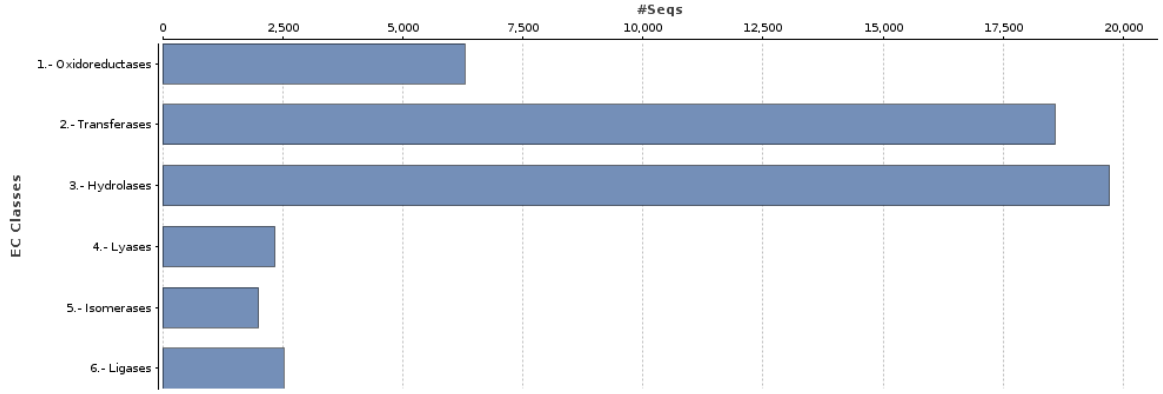

Fig. A3.1 Enzyme code distribution of the merged *P. oceanica* transcriptome.

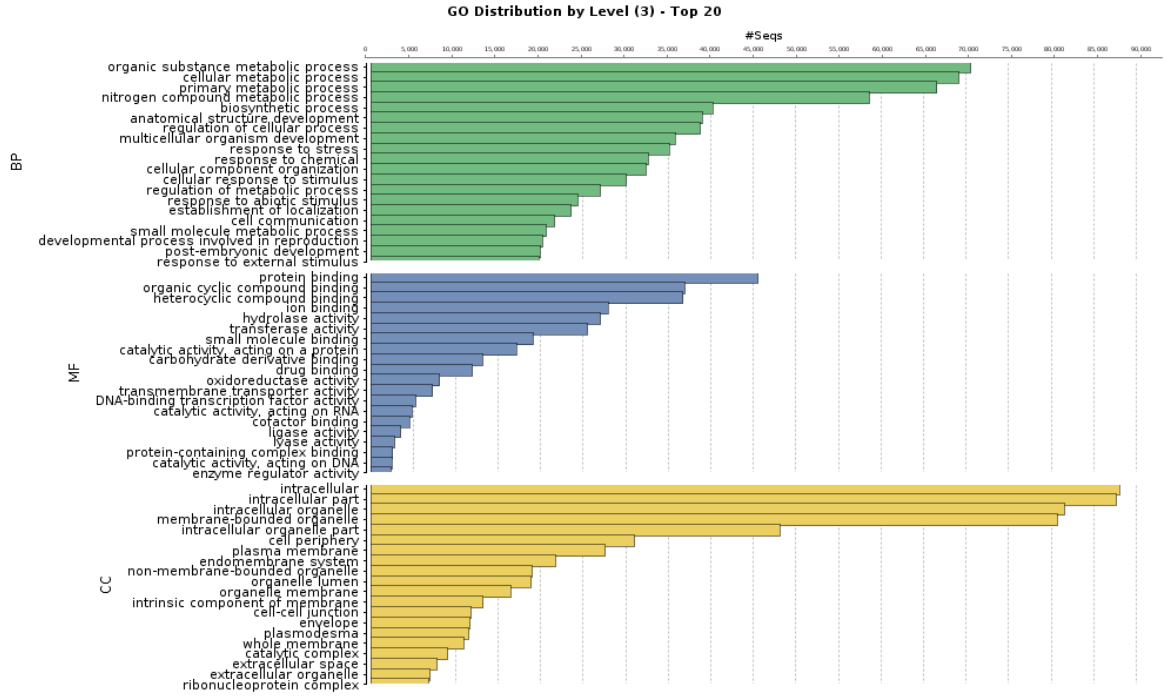

Fig. A3.2 Top 20 GO term distribution as biological processes (BP), molecular functions (MF) and cellular components (CC) retrieved for the merged *P. oceanica* transcriptome.

Table A3.3 Full list of DEGs associated to photosynthesis, light harvesting, chlorophyll biosynthesis and Calvin cycle in the contrasts MP_LL-MP_C (MP) and MO_LL-MO_C (MO) (FC > ±2; FDR <0.05). Transcript name, fold expression change (logFC) and FDR value are given.

Description	logFC (MP)	FDR	logFC (MO)	FDR
Photosystem I subunit O	-3.2	1.38E-05	-2.7	2.98E-03
Photosystem I reaction center subunit III, chloroplastic	-2.1	4.10E-02	-2.8	1.28E-02
Photosystem II reaction center W protein, chloroplastic	-2.8	2.22E-02		
Photosystem I reaction center subunit IV, chloroplastic			-1.1	8.79E-03
Photosynthetic NDH subunit of subcomplex B 4, chloroplastic	-2.3	4.84E-02	-3.2	1.90E-02
Photosynthetic NDH subunit of subcomplex B 4, chloroplastic	4.2	4.87E-02	-6.2	2.13E-02
Photosynthetic NDH subunit of subcomplex B 4, chloroplastic	6.0	3.48E-02		
NAD(P)H-quinone oxidoreductase chain 4, chloroplastic	-2.7	7.26E-03		
Protein LOW QUANTUM YIELD OF PHOTOSYSTEM II 1	-2.3	2.89E-04		
Protein LOW QUANTUM YIELD OF PHOTOSYSTEM II 1			-1.6	2.40E-03
Protein LOW PSII ACCUMULATION 3, chloroplastic			-1.5	1.00E-02
RNA polymerase sigma factor sigE, chloroplastic/mitochondrial			-2.7	6.28E-03
Thiol-disulfide oxidoreductase LTO1			-0.6	1.14E-02
Chlorophyll a-b binding protein 8, chloroplastic	-2.2	3.43E-04		
Photosystem I chlorophyll a/b-binding protein 5, chloroplastic			-0.7	4.85E-02
Magnesium-protoporphyrin IX monomethyl ester	-2.7	1.75E-06	-2.4	4.12E-05
Tetrapyrrole-binding protein, chloroplastic	-2.0	3.30E-02		
Tetrapyrrole-binding protein, chloroplastic			-1.8	3.60E-02
Chlorophyll synthase, chloroplastic			-0.5	1.47E-02
Geranylgeranyl diphosphate reductase, chloroplastic			-0.9	3.30E-02
Lycopene epsilon cyclase, chloroplastic			-0.5	3.63E-02
Ribulose bisphosphate carboxylase/oxygenase activase 1, chloroplastic	-2.7	3.04E-06	-2.0	1.96E-04
Ribulose bisphosphate carboxylase/oxygenase activase 2, chloroplastic	-2.7	1.03E-04		

Ribulose bisphosphate carboxylase/oxygenase activase 2, chloroplastic			-1.7	7.44E-03
RuBisCO large subunit-binding protein subunit alpha, chloroplastic			-0.7	3.42E-02
Fructose-1,6-bisphosphatase, chloroplastic	-2.9	3.05E-03		

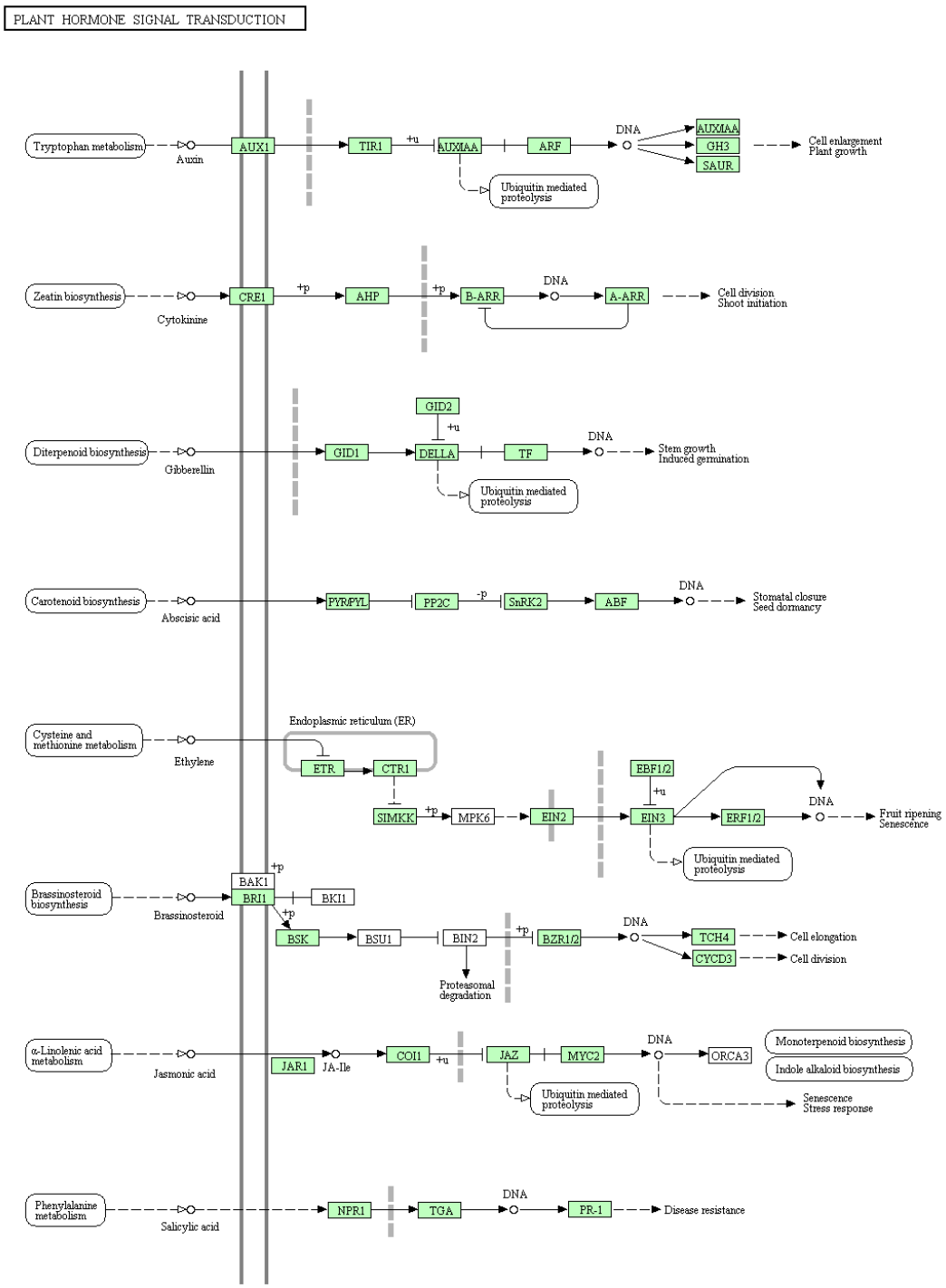

Fig. A3.3 Graphic depiction of the KEGG pathway "plant hormone signal transduction". In green are genes identified in the new assembled *P. oceanica* transcriptome.

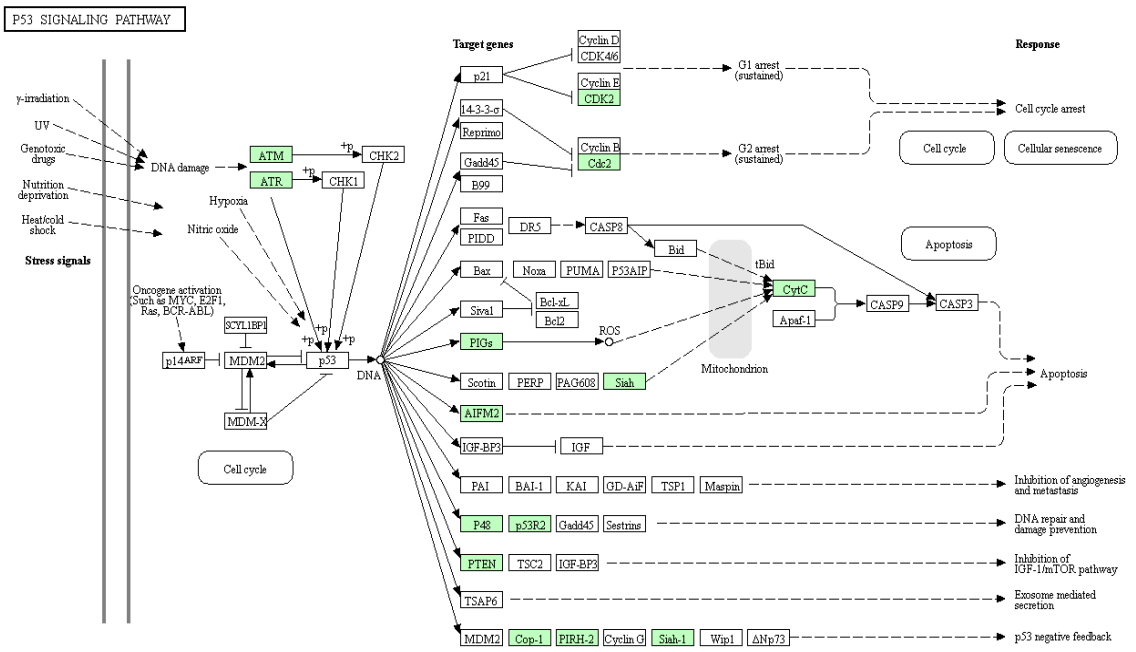

Fig. A3.4 Graphic depiction of the KEGG pathway "p53 signaling pathway". In green are genes identified in the new assembled *P. oceanica* transcriptome.

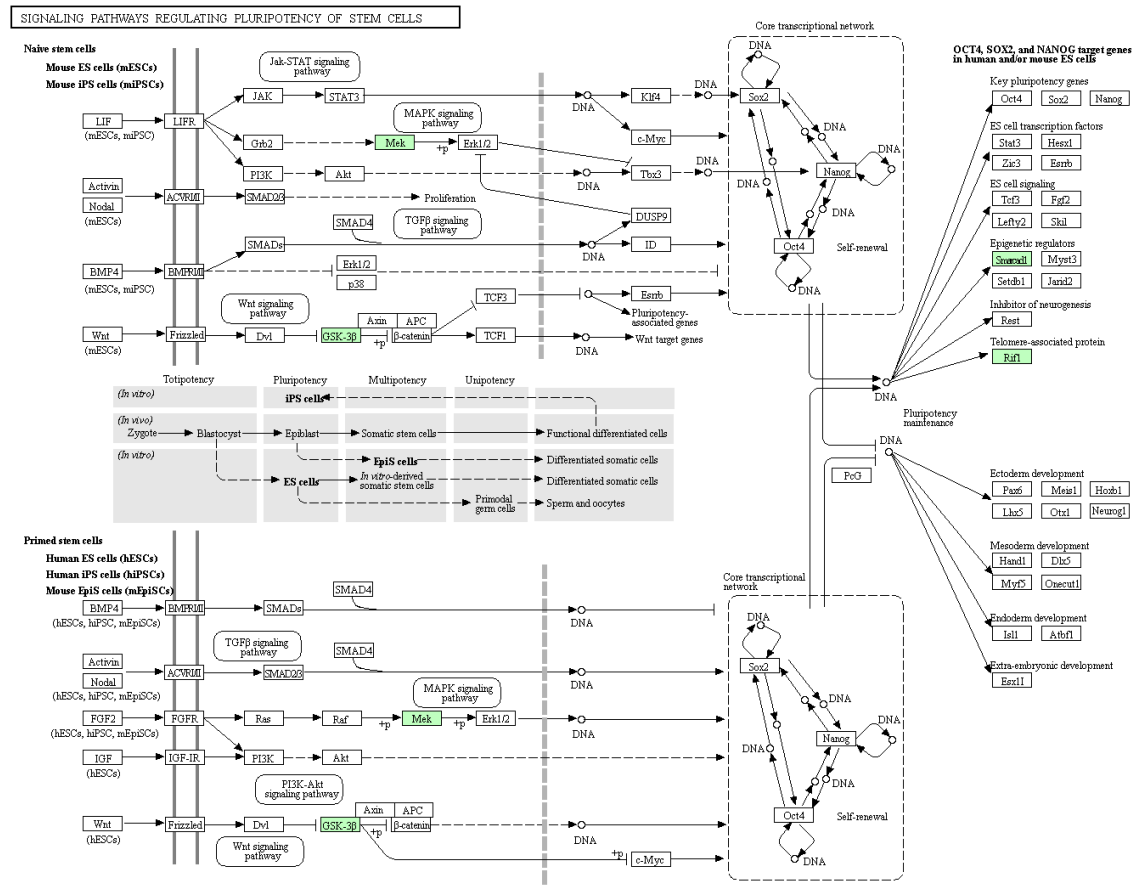

Fig. A3.4 Graphic depiction of the KEGG pathway "signaling pathway regulating pluripotency of stem cells". In green are genes identified in the new assembled *P. oceanica* transcriptome.

Chapter IV – Molecular level responses to multiple stressors in *Posidonia oceanica*: effects of herbivory and variable regimes of nutrient loading

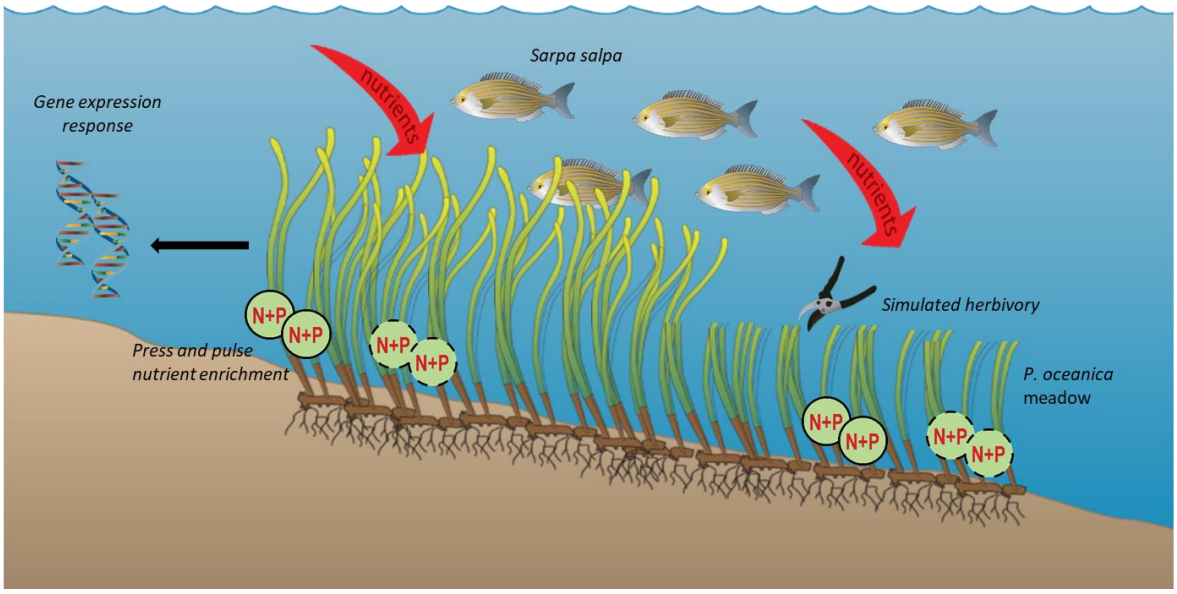

Fig. 4.2 Conceptual diagram of the experiment presented in this chapter. Effects of pulse *vs.* press nutrient loads and simulated overgrazing on *P. oceanica* gene expression. (All symbols taken from http://ian.umces.edu/imagelibrary/).

The work presented in this chapter has been published previously:

Miriam Ruocco, Lázaro Marín-Guirao, Chiara Ravaglioli, Fabio Bulleri, Gabriele Procaccini (2018) Molecular level responses to chronic versus pulse nutrient loading in the seagrass *Posidonia oceanica* undergoing herbivore pressure. Oecologia 188:23

The work was the result of a collaboration and only molecular data provided by MR are presented here. All other physiological, biochemical and morphological data to which this work refers have been published in Ravaglioli et al. (2018).

4.1 Introduction

***4.1.1** Mechanisms of nitrogen (N) assimilation and defense against herbivores in terrestrial higher plants*

N uptake and assimilation

Nitrogen (N) is an essential macronutrient for plant growth and development, and a building block of fundamental biological molecules, such as chlorophyll, amino acids, nucleic acids, and secondary metabolites. Most terrestrial plants absorb N from the soil, either in inorganic (e.g. nitrate and ammonium) and organic forms (e.g. urea, amino acids, peptides, and proteins). Nitrate ($NO3^-$) and ammonium ($NH4^+$) are the preferred N sources for higher plants (Wang et al. 2012; Krapp 2015). Uptake, assimilation, translocation, recycling and remobilization of N compounds are highly regulated and integrated processes that ensure an adequate supply of nutrients in a variable environment. Most of these steps are controlled by sophisticated gene regulatory networks acting both cell-autonomously and systemically (Vidal et al. 2010; Krapp 2015).

N research has largely focused on $NO3^-$ uptake, transport, and responses, as in addition to its role as a nutrient, $NO3^-$ can act as a signal that modulates nitrate assimilation-related gene expression, and ultimately plant growth and development (Wang et al. 2012). Nitrate is generally absorbed in the roots and mobilized to other organs by $NO3^-$ transporters. Two nitrate influx systems are present in plants, namely the high-affinity transport system (HATS), consisting of either inducible and constitutive components, and the low-affinity transport system (LATS) (Crawford and Glass 1998). Within these two systems, four different families of $NO3^-$ transporters have been characterized (i.e. NPF, NRT2, CLC, and SLAC/SLAH), each one comprising a large number of genes (Léran et al. 2014), displaying quite specific functions and strongly regulated at the transcript level by internal and external cues (O'Brien et al. 2016). Membrane-bound transporters are required for nitrate uptake from the soil but also for inter- and intracellular movements of nitrate inside the plants (Wang et al. 2012). After uptake, nitrate can either be metabolized directly in the roots, or transferred via xylem vessels to aerial parts of the plant and assimilated in the shoots (Andrews 1986). Nitrate is then reduced in the cytosol to nitrite by Nitrate reductase (NR), and transported into the chloroplast (plastids in roots) to be further reduced to ammonium by Nitrite reductase (NiR). These two enzymatic steps are costly in terms of reducing equivalents (from NADPH and ferredoxin), and thus tightly regulated. Ammonium is finally incorporated into aminoacids (glutamine/glutamate) by Glutamine synthetase (GS)/Glutamine-2-oxoglutarate

aminotransferase (GOGAT) cycle. N assimilation is responsive to internal and external clues including N metabolites. Regulation of assimilation enzymes occurs at the levels of transcription, translation, and posttranslational modification (O'Brien et al. 2016).

Plant defense strategies in response to herbivory

Plants can respond to herbivory either by reducing herbivores preference (e.g. synthesizing toxic chemicals and reducing leaf nutritional quality) (i.e. resistance traits) or diminishing the negative effects of consumption on fitness-related traits (e.g. altering physiological processes such as photosynthesis, growth, phenology, and nutrient storage) (i.e. tolerance traits) (Núñez-Farfán et al. 2007; Zhou et al. 2015). These mechanisms are not mutually exclusive, rather a mixed strategy of defence, with the simultaneous allocation of resources to tolerance and resistance, is pervasive among most host plants (Núñez-Farfán et al. 2007). A wide range of morphological, biochemical and molecular adaptations that can be regulated in response to consumers (Tiffin 2000; War et al. 2012; Sánchez-Sánchez and Morquecho-Contreras 2017). Specifically, plant resistance can occur via direct and indirect mechanisms that may be present constitutively or induced following the damage (i.e. induced resistance) (Howe and Jander 2008). Direct defence is mediated by plant morphological characteristics such as physical barriers (e.g. leaf surface wax, trichomes, leaf toughness and lignification) or production of chemical compounds (e.g. terpenoids, alkaloids, phenols and flavonoids) that either kill or retard the development of herbivores. Indirect defence is instead generally afforded through the emission of plant volatiles that attract natural enemies of herbivores (e.g. predators) (Howe and Jander 2008). The combination of direct and indirect defence provides resistance to a broad spectrum of herbivores in natural ecosystems. Changes in gene expression underlie the synthesis of most defensive secondary metabolites through the activation of specific biosynthetic pathways. However, herbivore attack can also lead to qualitative and quantitative changes in proteins playing a role themselves, such as plant lectins and chitinases, protease inhibitors (PIs), or antioxidative enzymes (e.g. peroxidases (PODs), polyphenol oxidases (PPOs), lipoxygenases (LOXs), catalase and superoxide dismutase) (Chen et al. 2005; Zhang et al. 2008; Gulsen et al. 2010; War et al. 2012). Synergistic interactions between plant defensive metabolites and proteins, that exert a combination of toxic and antifeedant effects, strengthen the host defence response.

Tolerance traits to herbivores include constitutive traits expressed before herbivory has occurred (e.g. those related to plant architecture such as high root/shoot ratio), and plastic phenotypic responses following the damage, such as compensatory growth, activation of dormant meristems, utilization/mobilization of stored reserves, increased photosynthetic

rate, and phenological changes like delayed flower and/or fruit production (Strauss and Agrawal 1999; Stowe et al. 2000; Tiffin 2000; Fornoni 2011).

In recent years, important advances in the identification of genes and pathways involved in plant resistance have been made (Zheng and Dicke 2008; Anderson and Mitchell-Olds 2011; War et al. 2012), driven by advances in genomic tools. In contrast, tolerance traits remains less characterized at molecular level (Schwachtje et al. 2006). Certainly, tolerance responses require the tuning of primary metabolism (mainly carbohydrates and nitrogen metabolisms) and related gene expression, for which signalling networks and molecular regulators have been only partially identified (Schwachtje and Baldwin 2008; Zhou et al. 2015).

4.1.2 *Effects of high nutrient loads and herbivore pressure on seagrasses*

In seagrasses, basic knowledge about nutritional physiology is much limited in respect to their terrestrial counterparts, as for the genetic makeup that contributed to adaptation to nutrients acquisition at sea. In general, seagrasses derive N from sediment pore water (mostly as $NH4^{+}$) and water column (mostly as $NO3^{-}$) (Touchette and Burkholder 2000b). The importance of leaves *vs.* roots in nutrient acquisition depends, in part, on the enrichment conditions and vary across species. However, N supply for most seagrasses is provided by leaf absorption from the water column (e.g. up to 50% in *T. testudinum* or 30-90% in *Zostera marina*, as reviewed in Touchette and Burkholder 2000b). This high nutrient uptake affinity of seagrass leaves can reflect their adaptations to oligotrophic environments.

While many seagrasses respond favorably to low or moderate nutrient enrichment, excessive anthropogenic-derived N loading can inhibit seagrass growth and survival, through direct and indirect effects (Orth et al. 2006; Burkholder et al. 2007; Ralph et al. 2007). Increased nutrient supply promotes the proliferation of fast-growing macroalgae, epiphytes, and ultimately phytoplankton (Duarte 1995), that compete with seagrasses for light and can cause seagrass die-off through shading (e.g. Hauxwell et al. 2003). High N availability can also have a negative effect on the plant itself, through direct ammonium toxicity (Van Katwijk et al. 1997), and increased internal energy demand and C skeletons for rapid ammonium assimilation, thus impairing seagrass productivity (Touchette and Burkholder 2000b; Invers et al. 2004). Finally, an increase in nutrient levels can lead to increased grazing, possibly through an augmented palatability of leaves and associated epiphytes (McGlathery 1995; Heck et al. 2006; Cebrian et al. 2009; Balata et al. 2010; Prado et al. 2010; Tuya et al. 2013). Marine coastal ecosystems can be exposed to either chronic elevation of nutrient levels or to abrupt, temporary increases in nutrient loading via river run-off after strong rainfall events.

The temporal patterns of fertilization events (i.e. chronic *vs.* pulse) on marine macrophytes has been only assessed by Murphy et al. (2012) and Tuya et al. (2015), in salt marshes and seagrasses, respectively. Tuya and co-authors found that chronic elevated nutrient supply reduced seagrass above-ground biomass, and increased pigment content more than pulse events, while there were no differences in photosynthetic performance related to the temporal pattern of fertilization. At molecular level, only Pernice et al. (2016) analysed the expression profiles of fundamental genes related to nutrient assimilation (GS/GOGAT cycle) in the seagrass *Z. mulleri* and correlated molecular data with the overall rate of nutrient uptake in above- and below-ground tissues. Moreover, a recent study revealed that nutrients enrichment might mitigate the negative impact of ocean acidification on *P. oceanica* also through molecular rearrangements, which include e.g. the up-regulation of N transporters genes and down-expression of antioxidants (Ravaglioli et al. 2017).

Grazing has been traditionally considered to be a natural disturbance with a relatively low impact on seagrasses (Short and Wyllie-Echeverria 1996), due to poor nutritional quality and high cellulose content of seagrass leaves that contribute to unpalatability (Duarte 1990; Hemminga and Duarte 2000). However, there is growing body of evidence that herbivory might be much more important than previously acknowledged in altering seagrass biomass, productivity and modulating species composition (Kirsch et al. 2002; Hughes et al. 2004; Tomas et al. 2005; Valentine and Duffy 2006). Intense herbivory events, due to natural or human-related activities (e.g. the establishment of marine protected areas), can be responsible for high leaf consumption rates that equal or exceed seagrass production rate (i.e. overgrazing), and contribute to seagrass decline (Orth et al. 2006; Eklöf et al. 2008a; Fourqurean et al. 2010; Christianen et al. 2014). In seagrasses, the induction of either tolerance and/or resistance traits has been observed, including compensatory growth, increase of photosynthetic rate, reallocation of energy and resources from undamaged to damaged tissue (Valentine et al. 1997b; Moran and Bjorndal 2005; Eklöf et al. 2008b; Verges et al. 2008; Sanmartí et al. 2014), and induction of chemical defence (Martínez-Crego et al. 2015). At molecular level, so far there are no studies addressing the remodelling of transcriptome following herbivory events, thus limiting our understanding of molecular basis of inducible defence strategies in seagrasses. On the other hand, the first genome sequencing of a seagrass species (i.e. *Z. marina*) revealed the disappearance or the drastic reduction of gene families associated to basal secondary metabolism, such as volatile compounds biosynthetic enzymes and sensors, including terpenoids and ethylene-related genes, and this has been related to the loss of stomata, through which they are emitted in terrestrial plants (Olsen et al. 2016). This is at odds with the fact that the marine environment

harbors just as many (albeit different) herbivores as on land, hence anti-herbivory defences in seagrasses may involve other pathways than those associated with terrestrial plants, but these remain to be identified.

Nutrient enrichment and grazing interact in a variety of ways (Burkepile and Hay 2006). Herbivores have been shown to offset the effects of eutrophication either by grazing epiphytic algae (Hughes et al. 2004; McSkimming et al. 2015) or increasing seagrass production and nutrient export (Christianen et al. 2012). On the other hand, nutrient availability may have an important role in determining plant's ability to compensate for herbivore-caused tissue losses (Verges et al. 2008), as previous studies have shown a significant translocation of nutrients stored in the rhizomes or among ramets in overgrazed plants (Valentine et al. 2004; Alcoverro and Mariani 2005; Tuya et al. 2013).

4.1.3 *The study*

The coupling of multiple stressors in marine systems can result in complex and unforeseen effects on organisms (Crain et al. 2008; Gunderson et al. 2016). Predicting and understanding the mechanisms underlying such interactions represent one of the most pressing problem in ecology and conservation (Gunderson et al. 2016). Most studies addressing this issue have focused on the mean effects of properties such as intensity and duration of stressors, however, variability in the distribution of stress events over time has been recognized as one of the key determinants of the overall effect of the disturbance regime (Benedetti-Cecchi et al. 2006; Molinos and Donohue 2010).

In seagrasses, a number of recent studies have investigated the simultaneous action of multiple (abiotic and biotic) disturbances (see Chapter I - 1.1.3) giving some insights on plant responses at different levels, from growth and survival to physiology. However, there is a lack of experiments manipulating multiple stressors and temporal patterns of disturbances. In addition, a mechanistic understanding of gene expression changes that accompany species acclimation to multiple stressors, and drive responses at higher level of organization, remains largely unexplored.

In this study, I investigated the individual and combined effects of anthropogenic-derived nutrient enrichment and simulated high grazing pressure on the seagrass *P. oceanica*, focusing on molecular level responses. In respect to works presented in previous chapters, addressing the short and medium-term plant stress response, here I wanted to explore the long-term rearrangements of *P. oceanica* metabolism upon acclimation to the growth conditions.

Specific aims included: I) to explore the molecular mechanisms underpinning the physiological response of *P. oceanica* to different intensity (i.e. control *vs.* high) and temporal patterns of nutrient loadings (i.e. chronic *vs.* pulse); II) to investigate the molecular basis of inducible defenses of plants to resist and tolerate high herbivore pressure; III) to assess the compounded effects of high nutrient loads and grazing disturbances on gene expression patterns, and specifically how plant defense strategies against herbivory vary depending on nutrient availability.

I tested the hypothesis that a temporary increase of nutrients in discrete events throughout the year could elicit a positive response of plants, resulting in the activation of the molecular machinery involved in nutrient assimilation. In contrast, a chronic increase of nutrients, simulating eutrophication, could suppress nutrient uptake and rather be detrimental for plants.

I adopted a candidate gene approach to detect the expression signature of specific genes involved in metabolic processes potentially affected by nutrient enrichment and herbivory, namely nutrient uptake and assimilation, photosynthesis and carbon fixation, oxidative-stress response and plant defense mechanisms. Yet, molecular data have been correlated to physiological and biochemical results from Ravaglioli et al. (2018) collected in the same experiment and time point.

4.2 Materials and Methods

Study area and experimental strategy

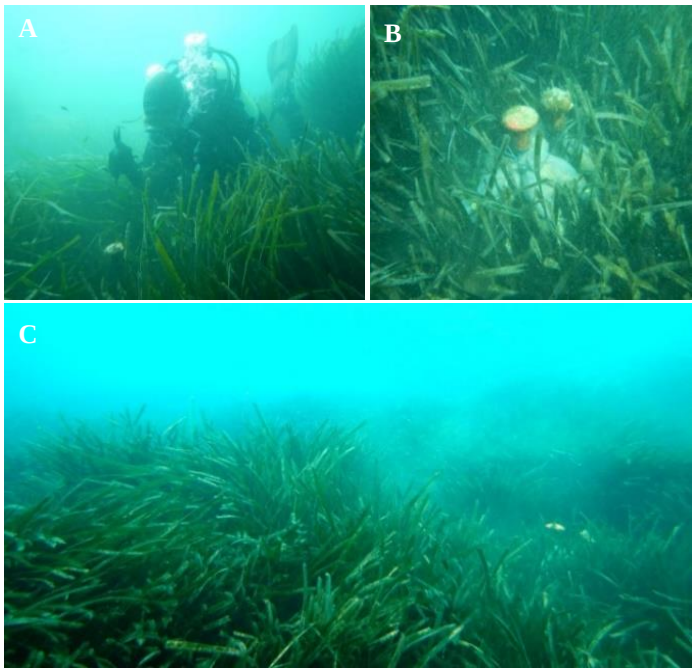

Fig. 4.2 (A) Simulation of herbivory by clipping leaf biomass; (B) bags with fertilizer used for nutrient enrichment; (C) *P. oceanica* meadow of Antignano (Livorno – Italy). Photo credit: G. Procaccini.

The individual and combined effects of nutrient enrichment and high herbivore pressure were evaluated by means of a manipulative experiment carried out from April 2015 to August 2016 within a dense *P. oceanica* meadow in a relatively pristine area south of Livorno (Antignano, Italy; 43°29'17.39''N, 10°19'33.17''E) (Fig. 4.2). Twenty-four plots (50x50 cm) were established at about 4 m depth and marked at their edges. Four plots were then randomly assigned to each of the six combinations of nutrients (control, press loading, and pulse loading) and herbivory (natural and high simulated herbivory) treatments (Fig. 4.3). Nutrient enrichment was simulated using Osmocote pellets (6 months controlled release fertilizer: 17:11:10 N:P:K), enclosed in plastic net bags (1 mm mesh size) fixed in the middle of the settled plots (Worm et al. 2000). Chronic nutrient enrichment was obtained through constant deployment of fertilizer (800 g) in each experimental plot across the duration of the experiment. Nutrient bags were replaced every two-months. On the contrary, to test the effects of pulse nutrient increase, the same total amount of nutrients used to generate the level of chronic nutrient enrichment, was distributed in five events through the experiment, that mirrored the natural distribution of heavy rains recorded in the study area (Servizio

Idrologico Regionale della Toscana; http://www.sir.toscana.it). Dissolved inorganic nitrogen (DIN) concentration was determined from water samples taken in each experimental plot, at 3 dates randomly chosen across the study period, using a continuous-flow AA3 Auto-Analyzer (Bran-Luebbe), following seawater standard analysis methods (Grasshoff et al. 2009) (see Fig. A4.1 in Appendix IV).

High herbivory was simulated by clipping all *P. oceanica* leaves within the established plots every 2-3 weeks, only during spring-summer months (May - August/September), where the maximum fish herbivory in *P. oceanica* is recorded (Tomas et al. 2005). We simulated the effect of overgrazing by the fish *Sarpa salpa*, which is the most important consumer of *P. oceanica* (Prado et al. 2007), by removing about 70% of leaf biomass. Seagrass leaves were cut to about 15 cm height, in respect to the beginning of the experiment where leaf length in control plots was 48.44 ± 2.69 cm (data from Ravaglioli et al. 2018). Natural herbivory plots were left uncaged, allowing natural grazing on *P. oceanica*.

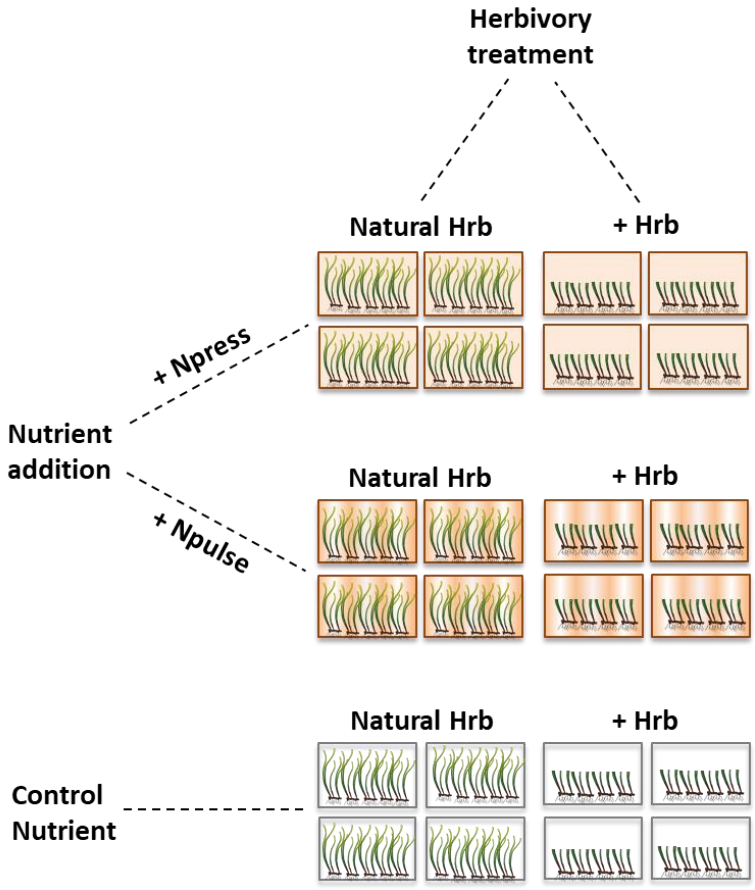

Fig. 4.3 Experimental design of the experiment. Four replicate plots were established for each of the six experimental conditions. +Npress = chronic/press nutrient loading; +Npulse = pulse nutrient loading; +Hrb = high simulated herbivory.

RNA extraction and cDNA synthesis

Gene expression analysis was carried out on *P. oceanica* leaf samples collected at the end of the experiment, when all nutrient enriched plots (both press and pulse) had been exposed to the same amount of nutrients. Only middle portions (ca. 5 cm) of mature leaves (rank 3) were taken. A leaf sub-sample × plot × treatment was collected by SCUBA diving, for a total of 24 samples (n=4). Plant material was entirely submerged in RNAlater© tissue collection (Ambion, life technologies) directly at sea, to keep storage time at minimum. Samples were then transported to the laboratory, stored for one night at 4°C, and finally stowed at -20°C until RNA extraction. Total RNA was extracted and quantity/quality checked as described in Chapter II (2.2). Five hundred nanograms from each RNA sample were retro-transcribed in cDNA as outlined in Chapter II (2.2).

Target gene selection

Primer pairs for the amplification of putative Reference Genes (RGs) and Genes of Interest (GOIs) were developed considering sequences from the *P. oceanica* published transcriptome (D'Esposito et al. 2017) using the software Primer3 v. 0.4.0 (Koressaar and Remm 2007; Untergasser et al. 2012) or selected from previous studies (Serra et al. 2012; Dattolo et al. 2014; Lauritano et al. 2015) (see Table 4.1). Design conditions included primer length (18-23 bp), T_m (~ 60°C), GC content (≥50%) and product size (100 to 200 bp). Increased photosynthetic activity as a mechanism of tolerance to compensate for biomass loss following herbivory attack has been well documented across numerous plant species. Accordingly, several genes involved in light reaction functions of photosynthesis (*psaC, psbA, psbD, PSBS* and *FD),* chlorophyll *a-b* binding proteins (*CAB-6A, LHCA4, CAB-151* and *LHCB4.2),* carbon dioxide fixation (*RBCS* and *RCA)* and chlorophyll biosynthesis *(POR*) were targeted. On the other hand, herbivore damage can also increase the resistance of the plant to further herbivore attack by inducing the synthesis of herbivore-deterrent metabolites (e.g. phenols) or defensive proteins. Hence, a number of genes encoding proteins involved in phenols metabolism (*PPO*) and antioxidative enzymes (*SOD, CAPX, APX* and *GR*) were also targeted. Finally, key genes involved in the first steps of nitrate assimilation in plant cells (*NRT2* and *NR*) were selected. In total, 6 putative RGs to be tested for stability in our experimental conditions and 19 GOIs were analysed.

Table 4.1 List of Reference Genes (RGs) and Genes of Interest (GOIs) assessed in *P. oceanica* using RT-qPCR. Gene and protein names, primer sequences, amplicon size (S, base pair), percent efficiency (*E*), correlation coefficient (R^2) and references, are given.

Gene	Protein	Primer Sequences 5'→3'	S	*E*	R^2	Reference
Reference genes						
18S	Ribosomal RNA 18S	F:AACGAGACCTCAGCCTGCTA R:AAGATTACCCAAGCCTGTCG	200	100%	0.99	Serra et al. 2012
eIF4A	Eukaryotic initiation factor 4A	F: TTCTGCAAGGGTCTTGACGT R:TCACACCCAAGTAGTCACCAAG	192	100%	0.99	Lauritano et al. 2015
EF1A	Elongation factor 1-alpha	F: GAGAAGGAAGCTGCTGAAAT R:GAACAGCACAATCAGCCTGAG	214	100%	0.99	Serra et al. 2012
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F:AGGTTCTTCCTGCTTTGAATG R:CTTCCTTGATTGCTGCCTTG	138	93%	0.99	Serra et al. 2012
UBI	Ubiquitin	F:CACCCTCGCTGACTACAACA R:TTTCTCAGCCTGACGACCTT	195	99%	0.99	Serra et al. 2012
L23	60s ribosomal protein L23	F:AAAGATACAGGCTGCCAAGG R:TGGTCCAACTTGTTCCTTCC	168	100%	0.99	Serra et al. 2012
Genes of interest						
psaC	Photosystem I iron-sulfur center	F: TCTTGGGATGGGTGTAAAGC R:AAGCTAGAGCCATGCTACGG	154	100%	0.99	This study
psbA	Photosystem II protein D1	F:GACTGCAATTTTAGAGAGACGC R:CAGAAGTTGCAGTCAATAAGGTAG	136	92%	0.99	Dattolo et al. 2014
psbD	Photosystem II protein D2	F:CCGCTTTTGGTCACAAATCT R:CGGATTTCCTGAGAAACGAA	161	100%	0.98	Dattolo et al. 2014
PSBS	Photosystem II 22 kDa protein	F:CCGCTCCTGTTGTTCTTCAT R:GGACCTCCTTCCTTGAGACC	158	100%	0.99	Dattolo et al. 2014
FD	Ferredoxin-1, chloroplastic	F:AGCATGGTAGCACCCTTCAC R:GGGGGAGGTATGAGAAGGTC	169	100%	0.99	Dattolo et al. 2014

RBCS	RuBisCO small subunit	F: CTGTACGCCCCTTTAATTCG R:TGACCAGGGAAGGTATCGAC	152	100%	0.99	Dattolo et al. 2014
RCA	RuBisCO activase, chloroplastic	F:TCAGACTGGGGGTAAGCAAC R:TCTACATCCTCGACCACTGC	187	100%	0.98	Marín-Guirao et al. 2016
CAB-6A	Chlorophyll *a-b* binding protein 6A, chloroplastic	F:CGACCGTTCTTGATCTCCTT R:AGTTCATCACCATCGCCTTC	154	96%	0.99	Dattolo et al. 2014
LHCA4	Chlorophyll *a-b* binding protein 4, chloroplastic	F:GGTCCAACACAACGTGACAG R:GACCTCCCTTGGAACCTTTC	200	100%	0.98	Dattolo et al. 2014
CAB-151	Chlorophyll *a-b* binding protein 151, chloroplastic	F:AAGCCCATTAGCACAACCTG R:GGGCAATGCTTGGTACTCTC	199	93%	0.99	Dattolo et al. 2014
LHCB4.2	Chlorophyll *a-b* binding protein CP29.2, chloroplastic	F:TCGAACACTTGACGGTGGTA R:ACGCTTCAGTTGGCTGAGAT	194	100%	0.98	Dattolo et al. 2014
POR	Protochlorophyllide reductase	F: AGTTCCACAGACGGTTCCAC R:AATCACCACCTGAGCGAGTC	194	98%	0.99	This study
SOD	Copper/zinc superoxide dismutase, cytosolic	F: GCTCCTGAGGATGAGATTCG R:AGGCCAATAACACCACAAGC	236	96%	0.99	Lauritano et al. 2015
CAPX	Ascorbate peroxidase, chloroplastic (stromal)	F: GCATGATGCTGGAACGTATG R:AATTTTGGGACCTCCAGCTT	228	100%	0.99	Lauritano et al. 2015
APX3	Ascorbate peroxidase 3, peroxisomal	F: TCAGCTTGCTGGAGTTGTTG R:CCCATGCGGTAAAAGATGTC	156	95%	0.99	Lauritano et al. 2015
GR	Glutathione reductase	F: AGTCCACACCAAATGGAAGC R:AAGGGGAGGGAAGGGTTATT	247	100%	0.99	Lauritano et al. 2015
PPO	Polyphenol oxidase	F: TTCTTTCCCTTCCACCATTG R:GGTGAGCTTGGGTTGGTAAA	149	100%	0.99	This study
NRT2	High-affinity nitrate transporter 2	F: AATCACCCAGCTCCTCATGC R:CAGCCCCGGTAGTTCTTGAG	246	95%	0.99	Ravaglioli et al. 2017
NR	Nitrate reductase	F: TAAGGCCATCCTTCCCTCTT R:CGGAGATTTGGCTGGTGTAT	142	90%	0.99	This study

Reverse Transcription-quantitative Polymerase Chain Reaction

RT-qPCR reactions were performed using Fast SYBR® Green Master Mix (Applied Biosystems) and Viia7 Real Time PCR System (Applied Biosystems) as described in Mazzuca et al. (2013). All RT-qPCR reactions were conducted in triplicate and contained a 1:50 dilution of the cDNA template (see Chapter II - 2.2 for further details). PCR efficiencies for all primer pairs were calculated as described in Chapter II (2.2). Primer's sequences, percent efficiencies (*E*) and regression coefficients (R^2) of RGs and GOIs are reported in Table 4.1. To normalize target gene-expression data, three different algorithms were utilized to identify the best RGs in our experimental conditions: BestKeeper (Pfaffl et al. 2004), geNorm (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004).

Data analysis

Relative quantification of gene expression was obtained with the same formulas outlined in Chapter II (2.2). Multivariate statistics was then used to assess the overall signal of all 19 GOIs. Specifically, a PERMANOVA was conducted on -ΔCT values with the Primer 6 v.6.1.12 & PERMANOVA + v.1.0.2 software package (PRIMER-E Ltd) (Clarke and Gorley 2006). The analysis consisted of two fixed factors: "Nutrients" (Nut) with three levels (chronic (+Npress), pulse (+Npulse) and ambient (Control)) and "Herbivory" (Hrb) with two levels (high (+Hrb), and natural herbivory (Natural)) and was conducted either using all the gene expression dataset or data grouped into functional categories (photosynthesis, carbon fixation, light harvesting and chlorophyll metabolism, plant defense, and N assimilation). A PCA of the complete gene expression dataset was also performed with the software PAST v.3.03 (Hammer et al. 2001) on -ΔΔCT values. A two-way ANOVA was then conducted on -ΔCT values to detect specific genes whose expression was affected by nutrient enrichment, herbivory or their combination. Normality and variance homogeneity of data were tested as in previous chapters. Student-Newman-Keuls post-hoc tests were used whenever significant differences were detected. Two-way ANOVAs were performed using the statistical package STATISTICA (StatSoft, Inc. v. 10). To explore how molecular information translate into physiological and morphological responses, a series of correlations analyses were performed using data collected in the same experiment and time point, and published in Ravaglioli et al. (2018). In details, the relationships between the expression of individual genes and photo-physiological parameters (effective quantum yield), pigments (Chl*a*, Chl*b*, total carotenoids) and secondary metabolites (phenols and flavonoids) content, and leaf growth rate, were investigated through Pearson's correlation analyses.

4.3 Results

Best reference gene (RG) assessment

A total of 6 putative RGs (see Table 4.1) were chosen and tested for stability in *P. oceanica* under nutrient enrichment and high herbivory. The two algorithms geNorm and NormFinder agreed in suggesting *eIF4A* and *UBI* as the best reference genes in our experimental conditions (see Tables 4.2 and 4.3), while the Bestkeeper approach indicated *L23* as the most stable gene based on the lowest SD of CT values (see Table 4.4). Since the use of reference genes belonging to different gene categories (i.e. biological processes) is highly recommended to avoid relatively large errors, I used all three best RGs (*eIF4A*, *UBI* and *L23*) for an accurate normalization of the target gene expression data.

Table 4.2 Expression stability of candidate RGs as calculated by geNorm in *P. oceanica*. Best candidate genes, with the lowest average expression stability, are underlined.

Gene name	Average expression stability (*M*)
eIF4A/UBI	0.48
GAPDH	0.64
L23	0.71
18S	0.95
EF1A	1.04

Table 4.3 Expression stability of candidate RGs as calculated by NormFinder in *P. oceanica*. Best candidate genes, with the lowest stability value, are underlined.

Gene name	Stability value	Standard error
UBI	0.22	0.08
eIF4A	0.27	0.08
GAPDH	0.40	0.08
L23	0.63	0.10
18S	0.72	0.12
EF1A	0.72	0.12

Table 4.4 Selection of reference genes in *P. oceanica* based on Bestkeeper. Lowest standard deviation (SD) of CT values is underlined.

Gene name	SD [± CT]
L23	0.52
GAPDH	0.73
UBI	0.97
eIF4a	0.92
18S	1.53
EF1A	1.53

4.3.1 Multivariate analysis of gene expression under high herbivory and nutrients

The PERMANOVA revealed a significant effect of the factor Hrb on the overall gene expression response (P <0.05), whereas the effects of the factor Nut, and the combination Nut×Hrb were not significant (Table 4.5). To evaluate if the two factors, individually or in combination, had an effects only on specific plant metabolic processes, the same analysis was conducted also on selected functional gene groups (photosynthesis, carbon fixation, light harvesting and chlorophyll metabolism, plant defense, and N assimilation) (Table 4.6). Herbivory significantly affected the expression of transcripts encoding for proteins involved in light reactions of photosynthesis (e.g. photosystems subunits) (P <0.05) (Table 4.6). In contrast, key genes for carbon fixation were significantly affected by the combination of nutrient enrichment and herbivory (P <0.05; Table 4.6). Subsequent pairwise tests (Nut×Hrb) indicated that at ambient nutrient level and under press nutrient supply, their expression differed between grazed and control plants ($P_{(MC)}$ <0.05), while under pulse fertilization, differences were not significant (Table 4.6). There was a significant interaction Nut×Hrb also on the expression of photosynthetic pigments-related genes (P <0.05) (Table 4.6). Specifically, only at ambient nutrient level, high herbivory caused a significant change in the transcriptional profile of genes for light harvesting proteins and chlorophyll biosynthesis ($P_{(MC)}$ <0.05) (Table 4.6), while at enhanced nutrient levels, there were no differences between herbivore treatments. Target genes with a role in plant defense (antioxidants and enzymes involved in phenols metabolism) were affected by the factor Hrb (P <0.05) (Table 4.6), whereas genes involved in N assimilation responded only to the factor Nut (P <0.05). Interestingly, there were significant differences between press and pulse treatments ($P_{(MC)}$ <0.05) and almost significant between press and control ($P_{(MC)}$ =0.08) (Table 4.6).

The PCA revealed a substantial separation between the experimental treatments (Fig. 4.4). Along the PC1 axis, which explains 51% of total variance, three main clusters can be identified: a first one including nutrient enrichment treatments (on the left side of the plot), a second one (in the middle), represented by grazed plants alone, and a third one in which nutrient enrichment treatments combined with high herbivory. Genes related to photosynthesis and carbon fixation (*PSBS*, *FD*, *RBCS*, *psbD* and *RCA*) were the most positively correlated with PC1 (Table 4.7). The component 2 (PC2), which explains the 31% of the total variance, mainly separates press and pulse nutrient fertilization, although differences were much weaker under high herbivory pressure. Pulse nutrient supply combined with natural or high herbivory was represented on the positive side of the axis, while press treatments were on the negative side. Genes contributing most to the PC2 were

those involved in nitrate uptake and assimilation: *NTR2* and *NR*, followed by *PPO* and photosynthetic-pigment related genes (*CAB-151* and *POR*). All of them positively correlated with the axis 2 (Table 4.7).

Table 4.5 Results of 2-way PERMANOVA conducted on -ΔCT to assess the overall contribution of all GOIs. $P_{(perm)}$ <0.05 are in bold, $P_{(perm)}$ <0.1 are underlined.

Two-way PERMANOVA				
Main test				
Source	**df**	**Pseudo-F**	$P_{(perm)}$	**Unique perms**
Nutrients	2	2.2009	0.0626	9951
Herbivory	1	4.4627	**0.0134**	9950
Nut×Hrb	2	2.0255	0.0886	9940

Table 4.6 Results of 2-way PERMANOVAs conducted on –ΔCT values for selected gene categories (photosynthesis, carbon fixation, photosynthetic pigments-related genes, plant defense and N assimilation). $P_{(perm)}$ <0.05 are in bold.

Two-way PERMANOVA

Photosynthesis

Source	df	Pseudo-F	$P_{(perm)}$	Unique perms
Nutrients	2	1.3035	0.2801	9953
Herbivory	1	5.7337	**0.0117**	9958
Nut×Hrb	2	0.91965	0.4357	9953

Carbon fixation

Source	df	Pseudo-F	$P_{(perm)}$	Unique perms	SNK pairwise tests
Nutrients	2	4.6274	**0.0218**	9932	+Npress: +Hrb ≠ Natural Hrb
Herbivory	1	9.6641	**0.0034**	9940	+Npulse: +Hrb = Natural Hrb
Nut×Hrb	2	3.636	**0.0392**	9959	Control: +Hrb ≠ Natural Hrb

Photosynthetic pigments-related genes

Source	df	Pseudo-F	$P_{(perm)}$	Unique perms	SNK pairwise tests
Nutrients	2	2.124	0.1254	9966	+Npress: +Hrb = Natural Hrb
Herbivory	1	2.3291	0.1265	9961	+Npulse: +Hrb = Natural Hrb
Nut×Hrb	2	3.2222	**0.0465**	9954	Control: +Hrb ≠ Natural Hrb

Plant defense

Source	df	Pseudo-F	$P_{(perm)}$	Unique perms
Nutrients	2	1.2711	0.2930	9949
Herbivory	1	3.4764	**0.0305**	9947
Nut×Hrb	2	0.96094	0.4598	9929

N assimilation

Source	df	Pseudo-F	$P_{(perm)}$	Unique perms	SNK pairwise tests
Nutrients	2	3.9382	**0.0186**	9957	Nutrients: +Npress ≠ +Npulse
Herbivory	1	2.2272	0.1227	9963	+Npress = Control (0.08)
Nut×Hrb	2	1.2323	0.3131	9949	+Npulse = Control

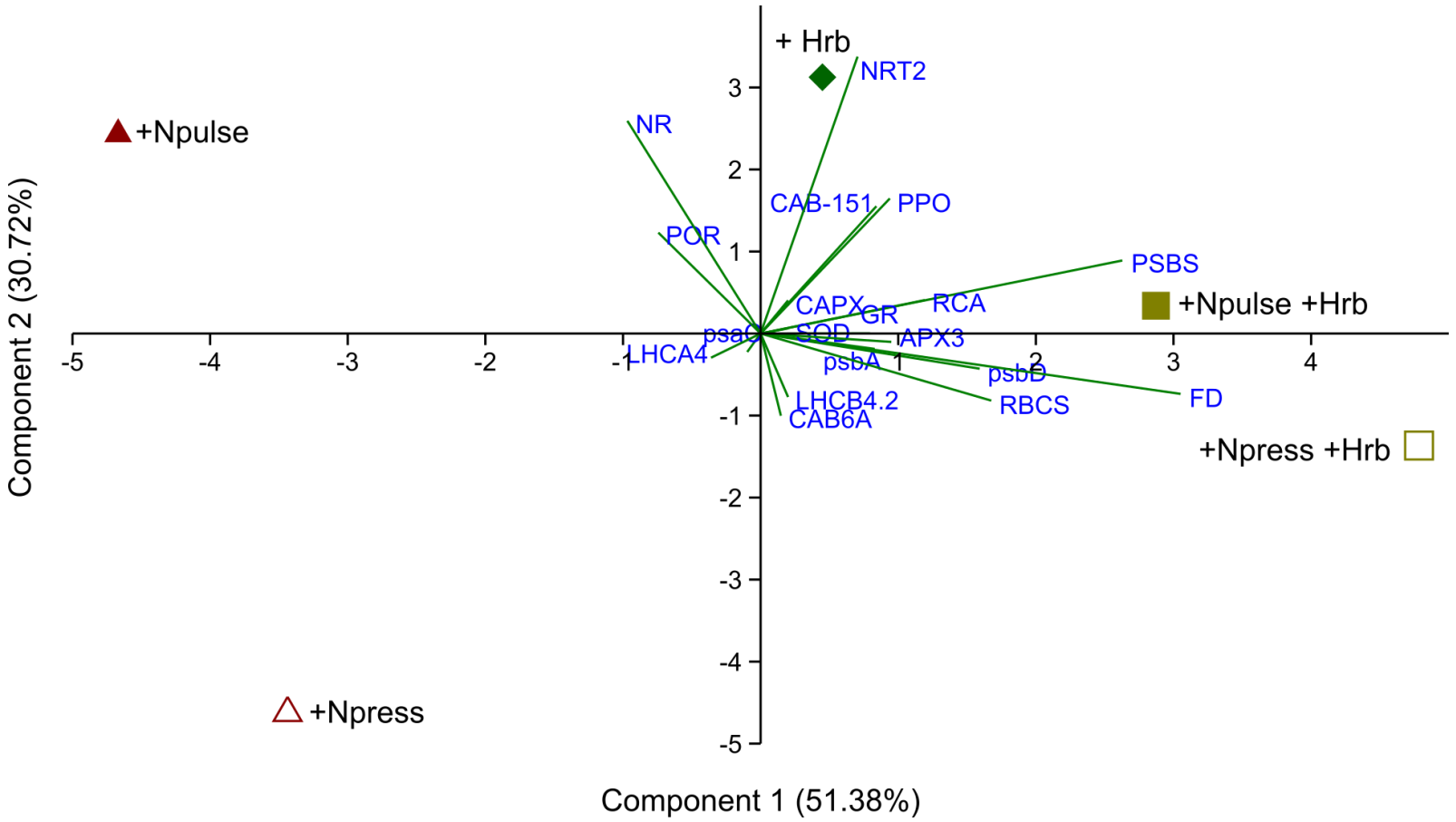

Fig. 4.4 PCA conducted using -ΔΔCT values for all 19 GOIs (+Npulse, +Npress, +Npulse +Hrb, +Npress +Hrb, +Hrb *vs.* control conditions (Control Nut, Natural Hrb)).

Table 4.7 Loadings of 19 GOIs on components 1 and 2 of the PCA. Loadings of genes contributing most to the principal components are in bold.

	PC1	PC2
psaC	-0.018	-0.042
psbA	0.154	-0.035
psbD	**0.295**	-0.080
PSBS	**0.488**	0.165
RBCS	**0.311**	-0.152
RCA	**0.221**	0.076
FD	**0.566**	-0.137
CAB6A	0.027	-0.186
LHCA4	-0.067	-0.055
CAB-151	0.156	**0.288**
LHCB4.2	0.037	-0.144
POR	-0.138	**0.228**
SOD	0.037	0.012
APX3	0.176	-0.019
GR	0.147	0.001
CAPX	0.037	0.075
NR	-0.180	**0.481**
NRT2	0.131	**0.626**
PPO	0.174	**0.305**

4.3.2 *Univariate analysis of gene expression under high herbivory and nutrients*

Ten out of the 19 GOIs were significantly affected by one of the factors or their combination (Table 4.8). Among genes whose expression was significantly altered by high herbivory there were those involved in light reaction functions of photosynthesis and photoprotection. Specifically, two key components of the photosystem II (PSII): *psbA*, encoding for the reaction protein D1, and *PSBS*, involved in non-photochemical quenching (NPQ), were significantly induced under high simulated compared to natural herbivory conditions (P <0.01; Table 4.8 and Fig. 4.5). Genes involved in the photosynthetic electron transport and carbon assimilation (Calvin cycle) were instead significantly over-expressed under both high herbivory and nutrient enrichment (either press and pulse), and these were Ferredoxin (*FD*) (P <0.05 for Hrb and Nut), RuBisCO small subunit (*RBCS*) (P <0.05 for Hrb and Nut), and RuBisCO activating enzyme (*RCA*) (P <0.01 for Hrb and P <0.05 for Nut) (Table 4.8 and Fig. 4.5). Only for two photosynthetic pigments-related genes a significant interaction

Nut×Hrb was found, and these were the enzyme Protochlorophyllide reductase (*POR*) which is involved in the pathway of chlorophyll biosynthesis, and a light harvesting protein (*LHCB4.2*). In line with the PERMANOVA results, only at ambient nutrient level both genes were up-regulated in grazed plants, in respect to natural herbivory-exposed ones, whereas under high nutrient loads (either press or pulse) differences were not significant (Table 4.8 and Fig 4.6). As hypothesized, also antioxidant enzymes were generally activated in response to high herbivory. However, only the transcript for Ascorbate peroxidase 3 (*APX3*) resulted significantly up-regulated (P <0.05; Table 4.8 and Fig. 4.7). Notably, also Glutathione reductase (*GR*) and Polyphenol oxidase (*PPO*) were affected by herbivory (albeit results were not significant; P <0.1) (Table A4.1 in Appendix IV and Fig. 4.7). Regarding genes related to N uptake and assimilation, High-affinity nitrate transporter 2 (*NRT2*) was up-regulated under high herbivory treatment (P <0.05; Table 4.8 and Fig. 4.8). In addition, there was a tendency for *NRT2* to increase under pulse nutrient supply and decrease under press treatment (SNK +Npress *vs.* +Npulse, P =0.07; Fig. 4.8). As expected, Nitrate reductase (*NR*), the key enzyme that catalyzes the first step of nitrate assimilation in plants, was affected by the factor Nut, but it showed a variable behavior according to the temporal variability of nutrient load. In particular, NR was significantly down-regulated under chronic fertilization in respect to control (P <0.05; Table 4.8 and Fig. 4.8), while under pulse nutrient supply (only when not combined with herbivory) it was up-regulated. Accordingly, the differences between press and pulse treatments were almost significant (P =0.056; Table 4.8 and Fig. 4.8).

Table 4.8 Results of two-way ANOVAs conducted on -ΔCT values. Only genes significantly affected by one of the factor or their combination are reported. For full results, see Table A4.1 in Appendix IV. *P* <0.05 are in bold, *P* <0.1 are underlined.

Two-way ANOVA					
	Effect	**df**	**F**	***P***	**SNK pair-wise tests**
Photosynthesis and photoprotection					
psbA	Nutrients	2	1.026	0.379	
	Herbivory	1	9.867	**0.006**	
	Nut × Hrb	2	0.347	0.712	
PSBS	Nutrients	2	1.084	0.359	
	Herbivory	1	9.741	**0.006**	
	Nut × Hrb	2	0.811	0.460	
FD	Nutrients	2	5.038	**0.018**	Nutrients: +Npress = +Npulse >
	Herbivory	1	7.262	**0.015**	Control
	Nut × Hrb	2	3.250	<u>0.062</u>	
Carbon fixation					
RBCS	Nutrients	2	4.383	**0.028**	Nutrients: +Npress = +Npulse >
	Herbivory	1	6.635	**0.019**	Control
	Nut × Hrb	2	3.167	<u>0.066</u>	
RCA	Nutrients	2	4.004	**0.036**	Nutrients: +Npress = +Npulse >
	Herbivory	1	14.842	**0.001**	Control
	Nut × Hrb	2	3.279	<u>0.061</u>	
Photosynthetic pigments-related genes					
LHCB4.2	Nutrients	2	4.178	**0.032**	+Npress: +Hrb = Natural Hrb
	Herbivory	1	3.517	<u>0.077</u>	+Npulse: +Hrb = Natural Hrb
	Nut × Hrb	2	3.783	**0.043**	Control: +Hrb > Natural Hrb
POR	Nutrients	2	0.991	0.391	+Npress: +Hrb = Natural Hrb
	Herbivory	1	1.229	0.282	+Npulse: +Hrb = Natural Hrb
	Nut × H	2	3.935	**0.038**	Control: +Hrb > Natural Hrb (0.07)
Antioxidants					
APX3	Nutrients	2	1.914	0.176	
	Herbivory	1	6.216	**0.023**	
	Nut × Hrb	2	0.137	0.873	
N assimilation					
NRT2	Nutrients	2	2.895	<u>0.081</u>	
	Herbivory	1	5.388	**0.032**	
	Nut × Hrb	2	1.967	0.169	
NR	Nutrients	2	3.730	**0.044**	Nutrients: +Npulse ≠ +Npress <
	Herbivory	1	0.026	0.873	Control
	Nut × Hrb	2	1.183	0.329	

Table 4.9 Pearson's correlation analyses between physiological, biochemical and growth data from Ravaglioli et al. (2018) and expression levels of individual genes. R coefficients and *P* values are shown (*n*=6). Significant values are indicated in bold.

	Chl *b*	Chl *a*	Carotenoids	Phenols	Flavonoids	ΔF/Fm'	Growth
psaC	0.6263	0.6016	0.5423	0.1733	0.1820	**0.8154**	-0.6142
	P=0.183	*P*=0.206	*P*=0.266	*P*=0.743	*P*=0.730	***P*=0.048**	*P*=0.195
psbA	0.4822	0.5035	0.4742	0.2564	0.2740	0.5067	-0.6477
	P=0.333	*P*=0.309	*P*=0.342	*P*=0.624	*P*=0.599	*P*=0.305	*P*=0.164
psbD	0.1650	0.1705	0.0124	0.6181	0.6471	0.3916	-0.4294
	P=0.755	*P*=0.747	*P*=0.981	*P*=0.191	*P*=0.165	*P*=0.443	*P*=0.395
PSBS	0.4641	0.5426	0.3042	0.3842	0.3589	**0.8118**	-0.6777
	P=0.354	*P*=0.266	*P*=0.558	*P*=0.452	*P*=0.485	***P*=0.050**	*P*=0.139
FD	0.6870	0.7461	0.4227	0.1844	0.1678	**0.8705**	**-0.8255**
	P=0.132	*P*=0.088	*P*=0.404	*P*=0.727	*P*=0.751	***P*=0.024**	***P*=0.043**
RBCS	0.6946	0.7419	0.4350	0.2008	0.1912	**0.8629**	**-0.8280**
	P=0.126	*P*=0.091	*P*=0.389	*P*=0.703	*P*=0.717	***P*=0.027**	***P*=0.042**
RCA	0.5660	0.6392	0.3230	0.3196	0.2967	**0.8635**	-0.7575
	P=0.242	*P*=0.172	*P*=0.532	*P*=0.537	*P*=0.568	***P*=0.027**	*P*=0.081
CAB6A	0.7210	0.7145	0.4190	0.2272	0.2501	0.7728	**-0.8254**
	P=0.106	*P*=0.111	*P*=0.408	*P*=0.665	*P*=0.633	*P*=0.072	***P*=0.043**
LHCA4	0.6645	0.6559	0.2838	0.2048	0.2226	0.7463	-0.7448
	P=0.150	*P*=0.157	*P*=0.586	*P*=0.697	*P*=0.672	*P*=0.088	*P*=0.089
CAB-151	0.5274	0.5895	0.1901	0.3746	0.3540	**0.8758**	-0.7147
	P=0.282	*P*=0.218	*P*=0.718	*P*=0.464	*P*=0.491	***P*=0.022**	*P*=.110
LHCB4.2	0.7194	0.7427	0.3903	0.1890	0.1946	0.8108	**-0.8381**
	P=0.107	*P*=0.091	*P*=0.444	*P*=0.720	*P*=0.712	*P*=0.050	***P*=0.037**
POR	0.4419	0.4818	0.0658	0.4121	0.3932	**0.8640**	-0.5872
	P=0.380	*P*=0.333	*P*=0.901	*P*=0.417	*P*=0.441	***P*=0.027**	*P*=0.220
SOD	0.5814	0.6303	0.3740	0.3277	0.3095	**0.9168**	-0.7226
	P=0.226	*P*=0.180	*P*=0.465	*P*=0.526	*P*=0.551	***P*=0.010**	*P*=0.105
CAPX	0.1552	0.2567	0.1312	0.5271	0.4583	**0.9252**	-0.3229
	P=0.769	*P*=0.623	*P*=0.804	*P*=0.283	*P*=0.361	***P*=0.008**	*P*=0.533
APX3	0.6362	0.7240	0.4734	0.1171	0.0956	0.7212	**-0.8180**
	P=0.174	*P*=0.104	*P*=0.343	*P*=0.825	*P*=0.857	*P*=0.106	***P*=0.047**
GR	0.5343	0.6146	0.5702	0.1330	0.0880	**0.8656**	-0.6152
	P=0.275	*P*=0.194	*P*=0.237	*P*=0.802	*P*=0.868	***P*=0.026**	*P*=0.194
PPO	0.0559	0.2492	0.1631	0.0168	-0.0969	0.5464	-0.1961
	P=0.916	*P*=0.634	*P*=0.757	*P*=0.975	*P*=0.855	*P*=0.262	*P*=0.710
NRT2	-0.2455	-0.0904	-0.3976	0.6426	0.5416	0.7154	-0.0047
	P=0.639	*P*=0.865	*P*=0.435	*P*=0.169	*P*=0.267	*P*=0.110	*P*=0.993
NR	-0.6743	-0.5507	-0.7516	0.4644	0.3579	0.2505	0.5617
	P=0.142	*P*=0.257	*P*=0.085	*P*=0.353	*P*=0.486	*P*=0.632	*P*=0.246

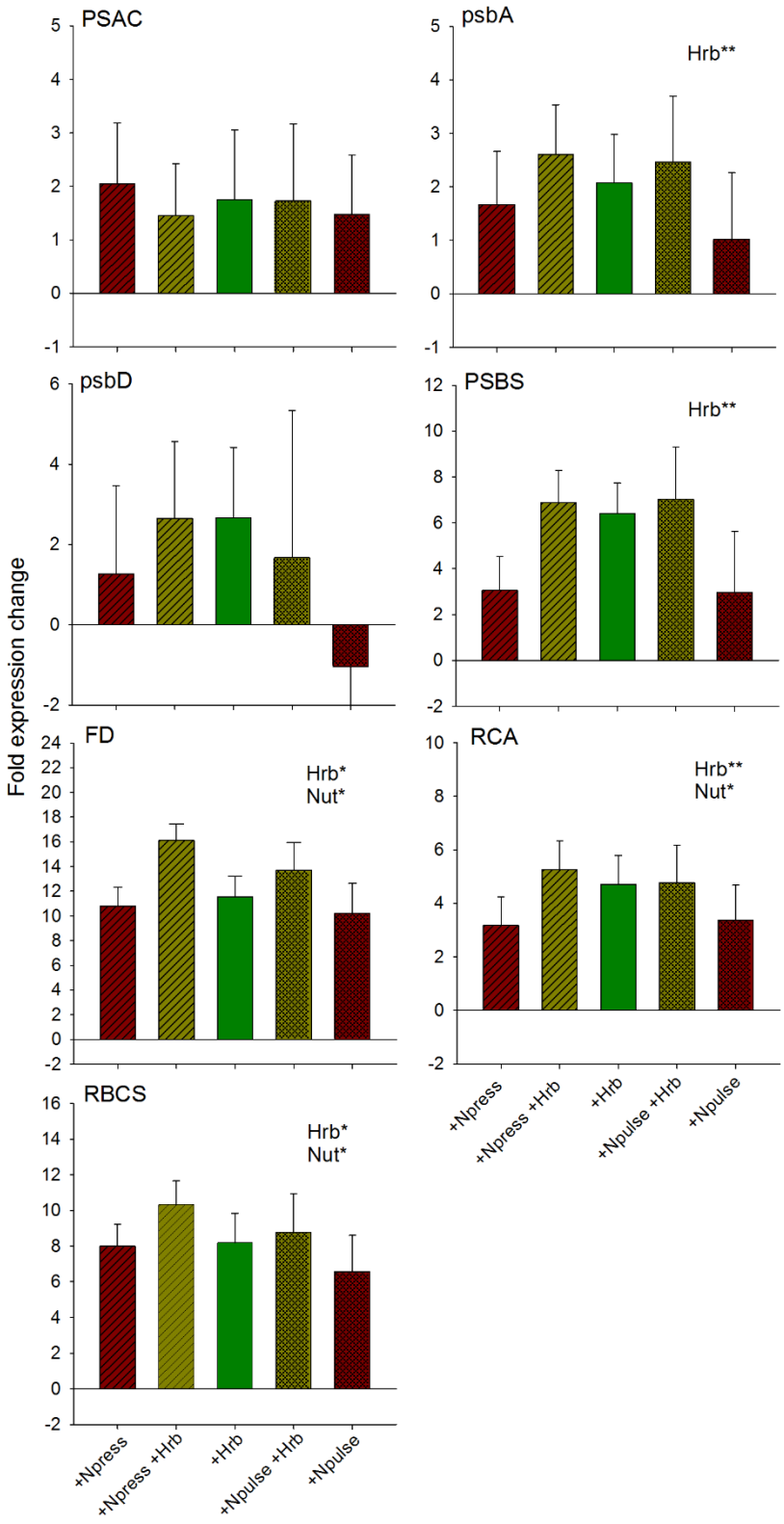

Fig. 4.5 Relative expression of photosynthesis and carbon fixation-related genes in +Npulse, +Npress, +Npulse +Hrb, +Npress +Hrb, +Hrb *vs.* control conditions (Control Nut, Natural Hrb) (x-axis) (mean +SE, *n*=4). ANOVA results are indicated on the top of the graphs. (*) *P* <0.05, (**) *P* <0.01.

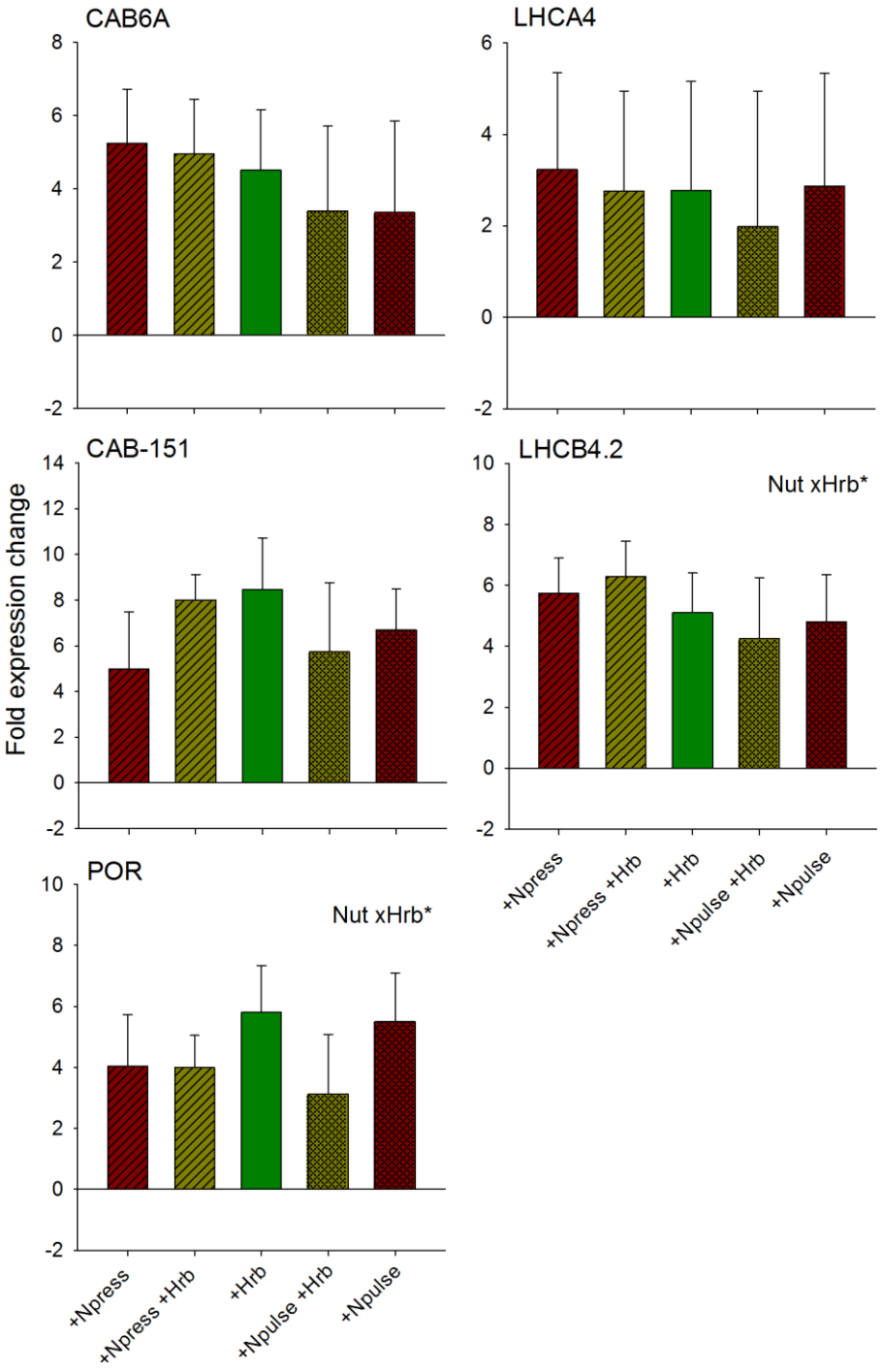

Fig. 4.6 Relative expression of light harvesting proteins and chlorophyll biosynthesis-related genes in +Npulse, +Npress, +Npulse +Hrb, +Npress +Hrb, +Hrb *vs.* control conditions (Control Nut, Natural Hrb) (x-axis) (mean +SE, *n*=4). ANOVA results are indicated on the top of the graphs. (*) *P* <0.05.

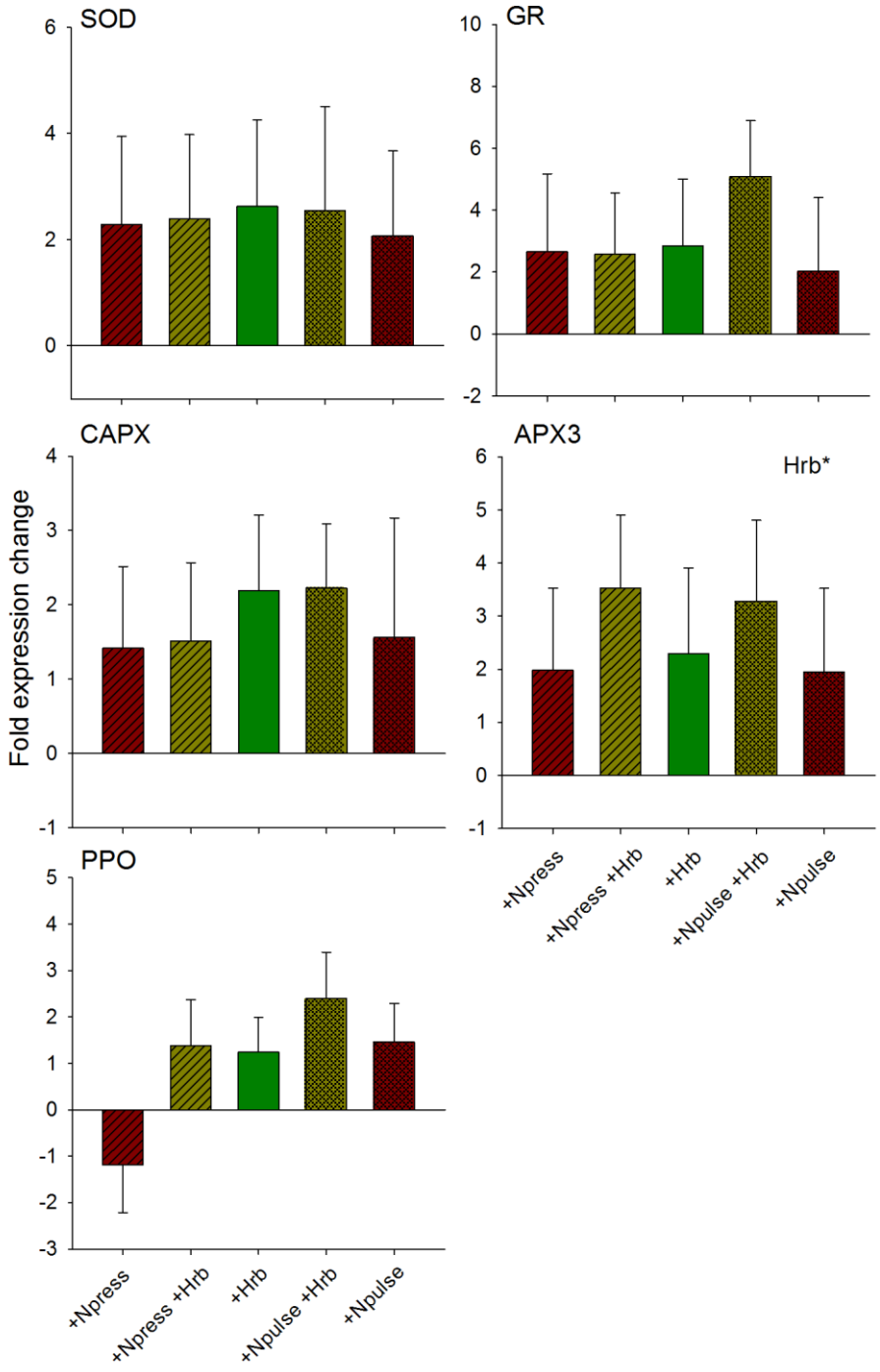

Fig. 4.7 Relative expression of plant defense-related genes (antioxidants and genes involved in phenols metabolism) in +Npulse, +Npress, +Npulse +Hrb, +Npress +Hrb, +Hrb *vs.* control conditions (Control Nut, Natural Hrb) (x-axis) (mean +SE, *n*=4). ANOVA results are indicated on the top of the graphs. (*) *P* <0.05.

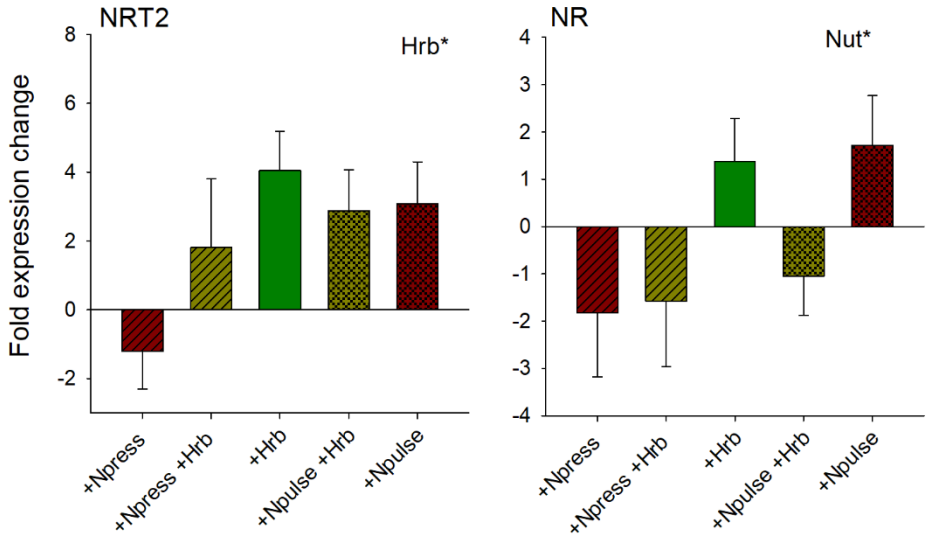

Fig. 4.8 Relative expression of genes involved in nitrate uptake and reduction in +Npulse, +Npress, +Npulse +Hrb, +Npress +Hrb, +Hrb *vs.* control conditions (Control Nut, Natural Hrb) (x-axis) (mean +SE, *n*=4). ANOVA results are indicated on the top of the graphs. (*) *P* <0.05.

4.4 Discussion

Overall, results show that long-term changes in *P. oceanica* metabolism following high simulated herbivory and fertilization, were orchestrated by complex transcriptional rearrangements of genes encoding for both primary and secondary metabolisms-related proteins.

Irrespective of nutrient availability, the drastic reduction in the photosynthetic surface area of plants subjected to intense herbivory pressure induced an increase in their photosynthetic efficiency through an increment in their photochemistry, electron transport and carbon fixation (Calvin cycle). This was reflected by the over-expression of specific photosynthesis-related genes, namely the photosystem component *psbA*, the electron carrier Ferredoxin (*FD*), and the gene for the Ribulose bisphosphate carboxylase/oxygenase small subunit (*RBCS*) with its activating enzyme RuBisCO activase (*RCA*). Only at ambient nutrient levels, highly grazed plants also induced the expression of chlorophyll *a/b* binding proteins (albeit only *LHCB4.2* significantly) and chlorophyll biosynthesis-related genes (*POR*), which improve light harvesting to support the augmented photosynthetic activity. Our results agree with photo-physiological data obtained in the companion study by Ravaglioli et al. (2018), indeed the expression of genes related to photosynthesis and light harvesting was significantly and positively correlated with plants' photochemical efficiency (i.e. effective quantum yield, ΔF/Fm') (Table 4.9), and this was interpreted as an induced tolerance mechanism for plants to compensate for biomass loss.

Tolerance to herbivores, i.e. the mechanisms that reduce negative effects of damage on plant fitness (Agrawal 2000; Stowe et al. 2000; Tiffin 2000; Núñez-Farfán et al. 2007), include constitutive traits expressed before herbivory has occurred (e.g. high root/shoot ratios), and plastic phenotypic responses following the damage, such as compensatory growth and activation of dormant meristems, mobilization of stored reserves, increased photosynthetic rate, and other phenological changes (Strauss and Agrawal 1999; Tiffin 2000; Fornoni 2011). Compensatory photosynthesis in remaining tissues following defoliation is a common physiological response to herbivory in terrestrial higher plants (Trumble et al. 1993; Strauss and Agrawal 1999; Thomson et al. 2003), and is often accompanied by increased expression of photosynthesis-related genes (e.g. Botha et al. 2006; Gutsche et al. 2008).

In seagrasses, although several studies have demonstrated the presence of tolerance strategies in response to herbivory, the molecular mechanisms behind these responses are yet to be identified. Previous studies measuring structural and physiological plant traits have highlighted the presence of compensatory responses, such as recruitments of new shoots

(Valentine et al. 1997a), or compensatory growth of existing shoots (Moran and Bjorndal 2005; Verges et al. 2008; Christianen et al. 2012; Sanmartí et al. 2014).

Our results also revealed the activation of photoprotective mechanisms under high herbivory, possibly due to the notable reduction of the canopy structure which attenuates plant self-shading and exposes middle and basal parts of the leaves to anomalous high light levels. Specifically, high simulated grazing triggered the accumulation of the transcript for the PSII subunit *PSBS* which, along with the presence of de-epoxidized xanthophylls, is important for photoprotective thermal energy dissipation (i.e. NPQ) (Niyogi et al. 2005; Demmig-Adams et al. 2014).

The increased photosynthetic activity, rather than representing a way to mitigate the effects of damage on plant fitness (i.e. tolerance trait), might be necessary to support the production of chemical defense (i.e. resistance trait), since the synthesis of defensive metabolites requires carbon fixation (Schwachtje and Baldwin 2008; Kerchev et al. 2012; Zhou et al. 2015). However, a concomitant allocation of resources to tolerance and resistance is likely, as demonstrated in most host plants in terrestrial environments (Núñez-Farfán et al. 2007). The presence of secondary metabolites, in particular phenolic compounds, that reduce the preference and/or performance of herbivores, is widespread in marine macrophytes and algae (Verges et al. 2007; Arnold et al. 2008; Arnold et al. 2014; Martínez-Crego et al. 2015; Zidorn 2016).

At ambient nutrient level, highly grazed *P. oceanica* plants tended to increase their leaf content of phenols and flavonoids (Ravaglioli et al. 2018), where they act as feeding deterrents to increase plant resistance. The oxidation of phenols catalyzed by Polyphenol oxidase (*PPO*) is an important defense mechanism in terrestrial plants against arthropod herbivores; quinones formed by these reactions bind covalently to leaf proteins, making them indigestible and thus decreasing plant nutritional quality (Bhonwong et al. 2009; War et al. 2012). Here, we did not detect a significant regulation of *PPO* enzyme in response to overgrazing, although there was a strong trend toward transcript over-expression (P =0.07). On the other hand, high herbivory significantly affected the expression of Ascorbate peroxidase (*APX3*). Other important anti-oxidative enzymes, such as *GR*, exhibited a general pattern of up-regulation, albeit not significantly. Induction and accumulation of antioxidant enzymes following herbivore damage or pathogen attack has been widely documented in recent years in terrestrial plants (Allison and Schultz 2004; Zhang et al. 2008; Usha Rani and Jyothsna 2010; Taggar et al. 2012). In particular, microarray experiments have revealed a number of genes associated with oxidative stress as up-regulated, including ROS scavengers (e.g. Ascorbate peroxidase and Catalase). The underlying hypothesis is that ROS

signals are integral to plant-herbivore interactions; they would be directly implicated in the induction of plant defense mechanisms against herbivores triggering enhanced expression of oxidative defense genes through signaling cascades (Kerchev et al. 2012).

High herbivore pressure also stimulates N uptake, as indicated by the over-expression of the gene for the high-affinity nitrate transporter *NRT2*, which is fundamental for plant nitrogen acquisition (O' Brien et al. 2016). This can reflect an attempt for *P. oceanica* to sustain growth and compensate for leaf consumption (Jaramillo and Detling 1988). However, plant amino acids also act as precursors of many defense compounds, hence this could also serve to support inducible production of defense metabolites (Zhou et al. 2015).

Nitrogen uptake and assimilation-related processes showed an opposite behavior when increased herbivory pressure was combined with fertilization. Both nutrient treatments (press and pulse) induced *NTR2* expression (nitrate uptake), but reduced that of *NR* (nitrate reduction). Under such combination, apparently, nitrate transport in the leaves is favored but nitrate reduction is declined. Because N stored in leaves is vulnerable to loss by defoliation, some terrestrial plants, actively accumulated nutrients in their roots upon leaf attack by herbivores (Frost and Hunter 2008; Erb et al. 2009; Millard and Grelet 2010). This change in nutrient allocation allows plants to withstand herbivory pressure, supporting regrowth or compensatory growth (Schultz et al. 2013). Basipetal or shoot-to-root N translocation have been described in several seagrass species (Touchette and Burkholder 2000a), therefore it is likely that nitrate absorbed by leaves in *P. oceanica* under high herbivory and nutrient loading, is transported and reallocated to other storage plant organs (i.e. rhizomes and/or roots) to make them inaccessible to aboveground herbivores. In this way, plants protect nitrogen reserves needed to regrowth, but also avoid an enhancement of the nutritional content of leaves that makes them more attractive to herbivores. Future work is needed to investigate the presence of induced changes in the expression of genes related to nutrient translocation and assimilation in rhizomes and roots to confirm this hypothesis. Upon fertilization (regardless the temporal variability of nutrient loading), *P. oceanica* plants increased significantly the abundance of the transcripts for two key enzymes related to carbon fixation, *RBCS* and *RCA*, likely to maintain their nutritional balance (C/N ratio). However, this seems not to be enough to compensate for the increase in N availability, as the leaf C/N ratio was significantly lower at the end of the experiment (Ravaglioli et al. 2018). It is possible that a significant proportion of photosynthetic electrons did not end up on the Calvin cycle to fix carbon. Alternatively, these electrons could have been diverted to reduce available nitrate for the formation of organic nitrogen compounds like amino acids. This is supported by the significant up-regulation of the transcript for the Ferredoxin (*FD*),

which, besides being a key component of the photosynthetic electron transport chain for the generation of reducing power, is also responsible of the diversion of electrons to other electron sink processes such as the nitrite reduction to ammonium, one the key steps of N assimilation (Fukuyama, 2004). Therefore, it seems that even with an increased photochemical efficiency and RuBisCO expression, fertilized plants experienced nutrient imbalance as a result of their long-term exposure to high nutrient levels (Burkholder et al. 2007), which could reflect in the negative effects on plants growth (Ravaglioli et al. 2018).

Interestingly, although all fertilized plants increased leaf nitrogen content (Ravaglioli et al. 2018), the response of genes involved in nitrate uptake and assimilation differed according to the temporal regime of nutrient loading (i.e. chronic *vs.* pulse), indicating the presence of sophisticated mechanisms to ensure adequate supply of nutrients in a variable environment, that act primarily at transcriptional level (Wang et al. 2012; O'Brien et al. 2016). In particular, nitrate transport (*NRT2*) and reduction (*NR*) related-genes were up-regulated in plants under pulse nutrient enrichment, whereas in the press treatment were both reduced. The first response could reflect an opportunistic behavior of *P. oceanica* to take advantage of the few nutrient enrichment events, as commonly observed in plants under nutrient limitation (Burkholder et al. 2007). The second response suggests the presence of a "saturation" behavior, to avoid excessive nitrogen uptake and assimilation once plant N requirement was fully covered by a long-term exposure to constant high nutrient levels. Nitrogen uptake and assimilation are, indeed, highly energy-requiring processes, where high quantities of reducing power for nitrate reduction and carbon skeletons for amino acids formation are required (Touchette and Burkholder 2000b).

Notably, the results presented above highlight the potential of using molecular biomarkers as indicators of nutrient enrichment status in seagrasses. For example, the expression of the enzyme Nitrate reductase has been proven to be affected not only by nutrient availability, as foreseeable, but also by temporal variability of nutrient loading. The enzymatic activity of Nitrate reductase was already suggested as a useful indicator of nutritional status in *Z. marina* (Roth and Pregnall 1988). Our observations suggest that also gene expression biomarkers can measure the long-term response of plant to differing nutrient levels, and may provide useful tools for nutrient impact assessment, as recently proposed for corals under thermal stress (Kenkel et al. 2014). Future research is needed, however, to properly test the suitability and applicability of these molecular signals as useful indicators of meadow eutrophication state, and in general of chronic seagrass stress (Macreadie et al. 2014).

In summary, high herbivore pressure affected the expression of several genes involved in plant tolerance and resistance traits (e.g. photosynthesis and plant defense mechanisms).

Genes modulating the response of plants to high nutrient levels were mostly those involved in carbon fixation and nutrient assimilation. Only for few genes, a significant interaction between herbivory and nutrient enrichment was detected (e.g. photosynthetic pigments-related genes category). Nonetheless, availability of resources seems to modify plant response strategies to herbivory, as the up-regulation of a N transporter gene was accompanied by the decline of Nitrate reductase transcript in the leaves, suggesting a change in nutrient allocation strategy. Finally, chronic and pulse nutrient supplies altered nitrate uptake and assimilation-related genes in a contrasting manner, suggesting that taking into account the temporal regime of nutrient loading is important to assess the physiological response of seagrasses to eutrophication.

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Appendix IV

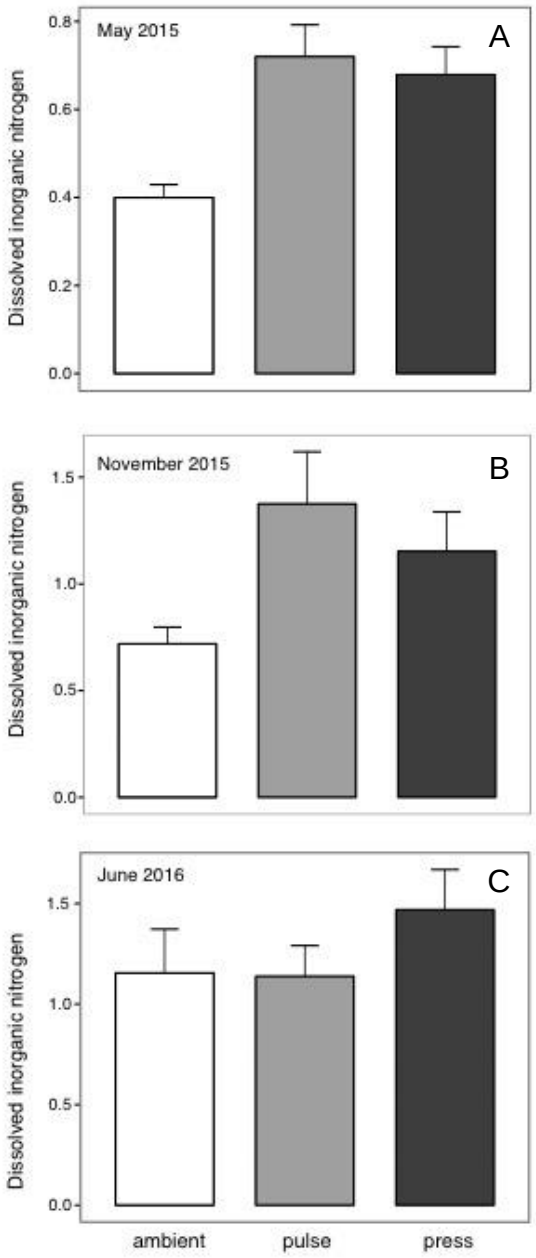

Fig. A4.1 Mean dissolved inorganic carbon (DIN) concentration (µmol L^{-1}, +SE, *n*=48 for May 2015 and June 2016; *n*=24 for November 2015) measured in the water column close to *P. oceanica* leaves on (A) May 2015, (B) November 2015 and (C) June 2016. Data from Ravaglioli et al. (2018).

Table A4.1 Full results of two-way ANOVAs conducted on -ΔCT values to assess the individual contribution of the 19 GOIs. *P* <0.05 are in bold, *P* <0.1 are underlined.

Two-way ANOVA				
	Effect	**df**	**F**	***P***
psaC				
	Nutrients	2	0.251	0.781
	Herbivory	1	0.166	0.689
	Nut×Hrb	2	0.694	0.512
psbA				
	Nutrients	2	1.026	0.379
	Herbivory	1	9.867	**0.006**
	Nut×Hrb	2	0.347	0.712
psbD				
	Nutrients	2	0.204	0.817
	Herbivory	1	2.512	0.130
	Nut×Hrb	2	0.067	0.935
PSBS				
	Nutrients	2	1.084	0.359
	Herbivory	1	9.741	**0.006**
	Nut×Hrb	2	0.811	0.460
FD				
	Nutrients	2	5.038	**0.018**
	Herbivory	1	7.262	**0.015**
	Nut×Hrb	2	3.250	0.062
RBCS				
	Nutrients	2	4.383	**0.028**
	Herbivory	1	6.635	**0.019**
	Nut×Hrb	2	3.167	0.066
RCA				
	Nutrients	2	4.004	**0.036**
	Herbivory	1	14.842	**0.001**
	Nut×Hrb	2	3.279	0.061
CAB-6A				
	Nutrients	2	1.641	0.222
	Herbivory	1	1.391	0.254
	Nut×Hrb	2	1.661	0.218
LHCA4				
	Nutrients	2	0.645	0.537
	Herbivory	1	0.116	0.738
	Nut×Hrb	2	1.141	0.342
CAB-151				
	Nutrients	2	0.985	0.393
	Herbivory	1	2.513	0.130
	Nut×Hrb	2	1.757	0.201
LHCB4.2				
	Nutrients	2	4.178	**0.032**

	Herbivory	1	3.517	0.077
	Nut×Hrb	2	3.783	**0.043**
POR				
	Nutrients	2	0.991	0.391
	Herbivory	1	1.229	0.282
	Nut×Hrb	2	3.935	**0.038**
SOD				
	Nutrients	2	0.875	0.434
	Herbivory	1	2.535	0.129
	Nut×Hrb	2	1.238	0.313
CAPX				
	Nutrients	2	0.327	0.725
	Herbivory	1	2.060	0.168
	Nut×Hrb	2	0.572	0.575
APX3				
	Nutrients	2	1.914	0.176
	Herbivory	1	6.216	**0.023**
	Nut×Hrb	2	0.137	0.873
GR				
	Nutrients	2	1.165	0.334
	Herbivory	1	3.324	0.085
	Nut×Hrb	2	0.921	0.416
PPO				
	Nutrients	2	2.850	0.084
	Herbivory	1	3.655	0.072
	Nut×Hrb	2	0.193	0.826
NRT2				
	Nutrients	2	2.895	0.081
	Herbivory	1	5.388	**0.032**
	Nut×Hrb	2	1.967	0.169
NR				
	Nutrients	2	3.730	**0.044**
	Herbivory	1	0.026	0.873
	Nut×Hrb	2	1.183	0.329

Chapter V – General discussion and conclusions

Fig. 5.1 *P. oceanica* meadow at Antignano (Livorno). Photo credit: Miriam Ruocco

5.1 General discussion

Human-induced environmental changes currently represent a major threat to marine biodiversity and ecosystem functions. Understanding the impacts on foundation species is critical for creating reliable predictions of the fate of entire communities that depend on them, and ultimately establish proper mitigation strategies (Bulleri et al. 2018). The unprecedented rate at which these changes are occurring implies species persistence will increasingly depend on the ability to respond and eventually adapt to novel environmental conditions (Bay et al. 2017). If adaptation capacity can be fast enough to keep up with rates of environmental change and how it varies among and within species, populations, and ecosystems, remain essential (and yet unresolved) questions in evolutionary biology (Merilä and Hendry 2014).

Among coastal ecosystems, seagrass meadows have largely recognized ecological and economic values. Their loss would compromise fundamental services, such as the support to commercial fisheries, sediment stabilization and carbon sequestration, among many others. Yet, the linkages between seagrass meadows and other habitats would be disrupted, producing much broader and long-lasting impacts than the loss of meadows themselves (Waycott et al. 2009). Worldwide awareness of the need for seagrass protection is growing, however if the management and regulation of direct threats such as dredging, anchoring or destructive fishing practices can be implemented relatively easily, much more difficult is to assess and eventually minimize the degradation caused by global processes, and the host of secondary changes they can give rise.

A more comprehensive knowledge of seagrass' tolerance capacity in face of current global and regional impacts (and notably their interaction), is imperative to forecast species' responses and persistence in the future ocean, and ultimately to establish proper conservation efforts. This knowledge starts with the exploration of molecular mechanisms underlying the cellular response to stress and driving plant responses at higher levels of organization (e.g. physiology and morphology).

The aim of this thesis was to explore how the stress response and the resulting acclimation capacity in seagrasses can vary at small scale, as a function of intrinsic plant features (e.g. the organ or tissue in question, or the shoot type), depending on the characteristics of the stressor/s in question, and when a combination of multiple stressors occurs. This complexity is often ignored when addressing the response of seagrasses to environmental changes, and this has major methodological implications, besides being of general interest in seagrass biology. Other fundamental levels of investigation, for example the variable effects of

stressors on different populations of the same species that can be locally adapted to the specific environmental settings, or the differential tolerance due to genotypic differentiation among individuals, have not been addressed in this thesis.

Here, the effects of main recognized abiotic (low light, high temperature and high nutrient levels), and biotic stressors (herbivory) were assessed in the Mediterranean seagrass *P. oceanica*, and acclimation strategies exhibited by the individual plants at molecular, photo-physiological and morphological levels were analyzed through different approaches.

The thesis started with the exploration of gene-expression gradients existing along the longitudinal axis of *Posidonia* leaves and among different leaves of the shoot, due to the presence of vertical and horizontal leaf-age gradients (**Chapter II**). Although this preliminary work does not directly address the response of seagrasses to any stressor (with the exception of natural variations in irradiance level within the canopy), it provides a basic framework to better understand how the stress response vary within and among leaves.

In terrestrial monocots, high-spatial resolution transcriptomic and proteomic studies have been used to define and characterize specific leaf developmental stages, and investigate the photosynthetic differentiation (e.g. Li et al. 2010; Mattiello et al. 2015). So far, these kind of molecular studies are completely absent in seagrasses, therefore the present data represent a first step toward a more comprehensive understanding of molecular reprogramming occurring across different leaf developmental stages responsible for variable photosynthetic capacity. The target gene-expression approach that was used in this study only allowed screening the behavior of a limited number of genes, involved in few metabolic pathways. In the next future, the use of high-throughput sequencing methods (e.g. RNA-Seq), which were not possible to apply for this experiment, could allow the detailed mapping of transcriptomic and proteomic changes occurring during seagrass photosynthetic development, the identification of signals that drive this process and the search for evolutionary differences in respect to terrestrial monocots.

The response of *P. oceanica* to heat stress was explored in the second part of the **Chapter II**. Through this study, I demonstrated for the first time the presence of a differential susceptibility and thermo-tolerance of leaf age sections. Previous studies have mainly addressed the effects of different abiotic stressors, including warming, on seagrass early life stages (i.e. seedlings) and have generally found a higher vulnerability, in respect to adult shoots (e.g. Olsen et al. 2012; Salo et al. 2014; Hernàn et al. 2016; Hernàn et al. 2017). These data show that a variability in the stress response (and thus in the acclimation capacity) at photo-physiological and molecular levels, is present also within adult *P. oceanica* shoots, along a single leaf blade. In particular, youngest leaf tissues, those fundamental for the

overall shoot growth, displayed the strongest photosynthetic inhibition, concomitant with the suppression of the PSII repair cycle, which could compromise their recovery capacity after the stress cessation. These results suggest that physiological and molecular evaluations conducted only on adult leaf tissues (e.g. intermediate sections of rank leaves 2 and 3) as common practice in seagrass research, would give unreliable estimates of the overall plant state, and should not be considered as a proxy for the whole shoot. Sampling a range of leaf age classes would perhaps yield the most representative tolerance measurements under stress events.

Another important consideration is that mortality of *P. oceanica* shoots were not noticed at the end of this experiment, despite the extremely high temperature (34°C) to which plants were exposed (but see below). The short duration of the exposure time would have possibly prevented mortality events to occur, however these results cast some doubts on the extinction predicted for the species by the middle of the 21th because of upcoming heatwaves (Jordà et al. 2012). The temperature rise simulated in this study was indeed much stronger than that recorded during the 2003-2006 heatwaves in the Mediterranean Sea, after which increased *Posidonia* mortality was described in natural populations (Díaz-Almela et al. 2009; Marbà and Duarte 2010). Similarly to my observations, Marìn-Guirao et al. (2018) did not detect *Posidonia* shoot mortality following a much longer exposure (six-week) to 4°C above the mean summer temperatures, and any observed negative effects on plant fitness (e.g. growth) disappeared after the stress event (i.e. recovery period). This seems to confirm the results of a long-term monitoring program of *P. oceanica* meadows in the warmest part of the western Mediterranean (Valencia region – Spain) for the period of 2002-2011 (Guillén et al. 2013). This long-term study indicated that most *P. oceanica* meadows were stationary or increasing their density and covering during the analyzed period, whereas no decline was observed. In conclusion, although clear negative effects of heat stress are detectable on *P. oceanica* at several levels of investigation, a general deterioration of its meadows cannot be attributable only to heatwaves, and shoot mortality observed in the field following these events, likely occurred due to the combination with local impacts (Guillén et al. 2013; Marìn-Guirao et al. 2018).

One of the aim of this thesis was to look for molecular bio-indicators, which could be used as a proxy of stress status in seagrasses (Macreadie et al. 2014). Notably, some target genes assessed in the experiment of **Chapter II** have a clear potential to be used as molecular biomarkers of heat stress, and likely of other stressors in *P. oceanica*. Specifically, two genes involved in the photosynthetic electron transport and carbon fixation, namely the ferredoxin (*FD*) and the small subunit of RuBisCO (*RBCS*), were among those exhibiting the strongest

variation in the level of expression under heat stress, more than other photosynthetic components (e.g. photosystem subunits). Importantly, these genes have shown a similar sensitivity also in other experiments, either with the same stressor (Marìn-Guirao et al. 2016) or in other context, for example when assessing the differential gene expression response of *P. oceanica* populations along a bathymetric gradient (i.e. shallow *vs.* deep) (Dattolo et al. 2014; Procaccini et al. 2017). Lastly, *FD* and *RBCS* were also strongly affected by nutrient enrichment and herbivory, as revealed in **Chapter IV**. This confirms the pivotal role of photosynthesis and related gene expression as a global stress sensor in seagrasses, and highlights that some components of this pathway, more than others, can be used as proxies of photosynthetic up or down-regulation following stress events. On the other hand, photosynthesis-related genes do not seem to be good candidates for discriminating among the effects of different abiotic/biotic stressors, since their transcriptional response appeared to be pervasive under several stressors, due to the key role of photosynthesis in plant energetic metabolism (Kosová et al. 2014).

Apart from photosynthetic-related genes, other two analysed genes showed great potential for future applications as molecular stress tools, namely Alternative oxidase 1a (*AOX*) and Bax inhibitor-1 (*BI*). Both genes showed a key role in mediating seagrass heat-stress acclimation, the former one minimizing ROS production across the mitochondrial electron transport chain and the latter preventing ROS-induced programmed cell death. Notably *AOX* sensitivity to different irradiance levels was already demonstrated in *P. oceanica* (Procaccini et al. 2017), and more recently its high responsiveness to high CO_2 was observed in *Cymodocea nodosa* (*personal observation*). The role of *BI* so far has been only demonstrated under heat stress (Marìn-Guirao et al. 2017; Traboni et al. 2018); however, its involvement in the acclimation of seagrasses to other stressors can be investigated in the future, since it regulates the process of programmed cell death, which represents one of the main mechanism of the cellular stress response. Another interesting result was that of the enzyme Nitrate reductase (*NR*) in **Chapter IV**. Its expression was proven to be differentially affected by chronic and pulse nutrient loads, and notably maintained in the long term (i.e. homeostasis response). Therefore, it could represent a valuable indicator of nutritional status in *P. oceanica* under meadow eutrophication state.

In the next future i) the proper validation of these candidate genes with *ad hoc* experiments in controlled and field conditions to establish their dose-response regulation, and ii) the development of user-friendly protocols to allow their use in a logistically feasible manner also for non-scientists, could be of great help for the early detection of seagrass stress status (Pernice et al. 2015). In this scenario, molecular indicators could significantly improve the

effectiveness of seagrass management strategies and conservation efforts under environmental changes (Macreadie et al. 2014).

Two main questions remain open: 1) which is the best approach to identify such responsive genes and 2) which is the most responsive/representative tissue/organ where to assess their expression.

Regarding the former question, both "target" and "omics" methods have pros and cons. Target approaches require some *a priori* knowledge on the metabolic pathways that could be affected by a certain stressor, but results coming from these "small-scale" studies are easier to interpret and, when the right target is selected, its expression level can be easily linked to a certain physiological process of interest. On the other hand, the screening power of high-throughput sequencing technologies is much wider, and information on any metabolic pathway involved in the response to the stressor in question can be acquired without any prior knowledge, but data analysis requires much longer time and technical efforts, especially in terms of bioinformatics competences. Both techniques have been used in this thesis, giving both meaningful results, however their applicability is also dependent upon the specific context and economic availability.

The second question was mainly addressed in **Chapter III**, where new transcriptome data have been generated from leaf tissues and shoot apical meristem (SAM) in *Posidonia*. This research shed first light on the stress response of organs, other than leaf, in seagrasses, and recognised the SAM as a key determinant for whole plant survival under light limitation. From these data emerged that SAM molecular response to stress occurred in a much greater extent in respect to leaves, revealing that it could really represent a primary stress indicator in seagrasses. It is worth mentioning that in the experiment of **Chapter II**, although shoot mortality was not detected under heat stress, meristem damages were actually noticed, where leaves were apparently still healthy. The lower tolerance threshold of the SAM and its fundamental role for whole plant organogenesis have to be carefully taken in consideration for future studies addressing seagrass stress response. If it will be further demonstrated that the molecular response of SAM to other abiotic/biotic stressors occurs not only in a greater extent, but also in much earlier than leaves, the role of these latter should be reconsidered and specific protocols to use this key plant organ as a monitoring tool, should be developed. These transcriptome data also increased considerably molecular resources available for future studies on seagrass evolutionary ecology and functional genomics. In particular, the sequencing of the SAM transcriptome offers great opportunities to explore how fundamental signaling pathways such as those involved in the regulation of stem cell pluripotency,

hormone biosynthesis and maintenance of meristem identity evolved and eventually differentiated in seagrasses, with respect to terrestrial angiosperms.

Another important aspect of the work presented in **Chapter III** was the first exploration of the differential molecular rearrangements occurring in plagiotropic *vs.* orthotropic shoots of *P. oceanica*, under light limitation. As discussed in previous chapters, clonal plants benefit from physiological integration among individual ramets, sharing resources and information. Clonal integration buffers against environmental changes and let the plant clone working as a "macro" organism. Under unfavourable conditions, resources can be transferred from one ramet to the others ensuring the whole clone survival. The molecular signals that regulate this phenomenon are completely unknown in seagrasses. Therefore, this represent the first comprehensive study giving some insights into the metabolism of different type of ramets within the clone, and it allowed the exploration of how their metabolic role can change under stress events. The underlying hypothesis is that, under stressful conditions, available resources could be transferred to apical shoots, in order to enable the colonization of new areas (escape strategy?), sacrificing resources from vertical ramets. Although I proposed some molecular mechanisms that could play a role in modulating resource sharing among ramets under light limitation, transcriptome data have to be explored much further, and specific experiments have to be designed in the future to confirm this hypothesis. For example, phytohormone (e.g. auxins and cytokinins) quantification could be carried out in different parts of the *Posidonia* clone, as well as the expression analysis of specific genes related to hormone biosynthetic pathways, hormone transporters etc., under control and stress conditions. Contemporary, the quantification of nutrients and carbohydrates should be performed, to demonstrate the actual movement/accumulation of resources in different ramets.

Finally, a significant aspect of this thesis was the recognition of the importance of epigenetic variations, primarily DNA methylation changes, as key mechanisms for phenotypic accommodation and adaptive responses to environmental changes in seagrasses. In **Chapter II**, changes in global DNA methylation level were identified across leaf developmental stages and in response to heat stress. These data confirmed that this epigenetic mechanism plays a role during both seagrass development and following stress events. However, an in-deep investigation of methylation targets and effectors (e.g. DNA methyltransferases) was not possible, due to the type of technique that was applied and the lack of genomic information in *P. oceanica*, limiting the functional interpretation of obtained results. In the future, the application of techniques recently developed for obtaining genome-wide methylation profiles also in non-model organisms (e.g. the reference-free reduced

representation bisulfite sequencing; Van Gurp et al. 2016) or the release of the *P. oceanica* genome would allow the screening of (putatively) all the differentially methylated sites (epigenetic polymorphisms) e.g. under control *vs.* stress conditions, their variations among and within populations etc. Moreover, the concomitant analysis of expression levels of different enzymes involved in DNA methylation or de-methylation and the relative quantification of differentially methylated genes would give a much more complete picture on the role of epigenetic variations in seagrasses.

In addition to DNA methylation changes, many transposable elements, in particular retrotransposons like those belonging to the *Copia* family have been identified as differentially expressed in the transcriptome of *P. oceanica* under low-light stress. Epigenetic mechanisms can adjust phenotypes or generate new phenotypes without modifying the DNA sequence, and sometimes these modifications can be transmitted across generations. On the other hand, the activity of transposable elements is known to be triggered by environmental cues, accelerating mutation rates and rewiring regulatory networks. Transposable elements and epigenetic components are intimately linked, potentially amplifying their actions on phenotypes and genotypes (Rey et al. 2016). Recent studies have shown that, different from the expectations, adaptive phenotypic responses of species and populations to environmental changes can be extremely rapid. A powerful molecular engine triggering such rapid phenotypic responses is most likely constituted by the interplay of these two mechanisms, which are sensitive to environmental stressors (Rey et al. 2016). Based on these observations, future studies should be aimed at characterizing transposable elements present in seagrasses and assessing their activity/mobility following stress events. In addition, the role of other epigenetic components such as histone modifications, histone variants and non-coding RNAs should also be investigated, as already carried out in other marine species (e.g. Gonzalez-Romero et al. 2017; Rodriguez-Casariego et al. 2018).

Ultimately, seagrasses could possess a hidden potential to fast adapt to current environmental changes, through the ecological advantages of clonal spread (e.g. resource and risk sharing) and the use of genetic and non-genetic components (e.g. transposable elements and epigenetics mechanisms) that facilitate and optimize phenotype variations in response to stress. As clonal plants they could particularly benefit from epigenetically regulated plasticity as an alternative to the slower mechanisms of adaptation based on genetic change (Douhovnikoff and Dodd 2015; Dodd and Douhovnikoff 2016). In addition, especially in long-living species such as *Posidonia*, epigenetic mechanisms could play a major role, since they can build through time (Douhovnikoff and Dodd 2015).

In this context, research in marine systems is lagging behind that of terrestrial systems; future studies, integrating the role of non-genetic mechanisms in modulating adaptive responses to environmental changes, will give a more holistic picture of seagrass evolutionary potential (Duarte et al. 2018), and perhaps provide reasons for fostering "**seagrass optimism**".

5.2 Conclusions

Main questions and findings addressed in this thesis are presented in the table below:

Table 5.1 Summary of the main questions addressed in this thesis.

Question	Chapter	Key findings
Does gene expression vary within and among seagrass leaves in natural conditions? *How gene expression modulates photo-physiological functions of specific leaf segments?*	**II**	The expression of key genes related to photosynthesis, chlorophyll biosynthesis, respiration, and PCD varied mostly along the leaf blades of *P. oceanica*, likely due to the strong vertical irradiance gradient present within seagrass canopy. Molecular changes paralleled photo-physiological variations existing from the base to the leaf tip, specifically in Fv/Fm, NPQ and r-ETR values. Among-leaf variations reflected mainly age differences of the leaf tissues. Most photosynthetic genes were more expressed in younger compared to older leaves, due to maturation processes and the establishment of the photosynthetic machinery. The induction of chloroplast and mitochondrial energy dissipation mechanisms together with the inhibition of PCD, orchestrate leaf photo-acclimatory responses. DNA methylation seems to play a role in modulating seagrass gene expression both across leaf development and during light acclimation.
Does the heat stress response vary within the same plant organ? *Can different leaf segments exhibit different thermo-tolerance?*	**II**	This study shows that the response to acute heat stress vary at fine spatial resolution within and among seagrass leaves. The vertical age gradient existing along *P. oceanica* leaves affected photo-physiological responses to heat stress more than leaf rank-differences. At gene-expression level, youngest leaf sections exhibited the strongest negative response to warming, suggesting a greater sensitivity of such tissues and lower thermo-tolerance. Besides a mild down-regulation of transcripts encoding for PSII subunits, youngest leaf portions exhibited an extreme down-regulation of key components of the photosynthetic electron transport, Calvin cycle, light harvesting complexes and chlorophyll biosynthetic pathways. On the contrary, the most up-regulated genes were those involved in energy dissipation and

		inhibition of PCD. A tendency for DNA hyper-methylation was observed under heat stress, but only in intermediate and oldest analyzed leaf sections.
Does the response to low-light stress vary between different shoot types?	**III**	This study shows that at photo-physiological and morphological levels, mild differences were observed in the response of plagiotropic and orthotropic shoots under low-light stress, although the reduction in shoot size and leaf growth rate at the end of the experiment were slightly higher for the former ones. On the contrary, whole transcriptome analysis revealed some differences between the two shoot types, where generally a restricted portion of DEGs was shared between plagiotropic and orthotropic shoots under LL. A greater number of DEGs was always associated to the LL response of plagiotropic shoots for both leaves and SAMs; however, orthotropic ones had a much more complex stress response, involving a higher number of biological processes. This highlighted for the first time at molecular level, a different metabolic role for apical and vertical shoots that deserves further investigations.
Is the shoot-apical meristem a better indicator than leaves of the whole plant status under low-light stress?	**III**	The number of DEGs and GO enriched biological processes identified in the response of shoot-apical meristems under LL was always higher than that identified for leaves. Enriched BP included fundamental functions related to the meristem maintenance and growth, organogenesis, as well as DNA damage/repair and cell proliferation. Notably, epigenetic-related processes were also among top-enriched processes. The most part of these enriched functions were not identified in the analysis of leaves. This revealed that the stress response starts primarily at the level of meristems, which are the most sensitive plant parts, with the lowest tolerance threshold. Meristem response occurs earlier in respect to leaves, therefore this open a new view, where the SAM-related response can be considered a fundamental indicator of seagrass status under stress.
Does seagrass react differently to continuous or episodic nutrient supply in their habitat?	**IV**	This study shows that the response of genes involved in N uptake and assimilation differed according to the temporal regime of nutrient enrichment. In particular, N transport and reduction related-genes were up-regulated in plants under pulse nutrient load, whereas in the chronic treatment were reduced. The first response reflects an opportunistic behavior of *P. oceanica* to take advantage of nutrient pulses when available, as observed in plants under nutrient limitation. The second response suggests the presence of a saturation behavior, to avoid excessive energy drain for N uptake/ assimilation once plant requirements were covered by the chronic exposure to high nutrient levels.
How seagrass respond to herbivory at molecular level?	**IV**	Intense herbivore pressure stimulated plants' photosynthetic activity, as demonstrated by the up-regulation of genes related to photochemistry, electron transport and carbon fixation. This

How the interaction between herbivory and variable regimes of nutrient loading affect seagrass molecular response?	is considered an induced tolerance mechanism to compensate for biomass loss. Availability of nutrients seems to modify plant response strategies to herbivory, as the up-regulation of a N transporter was accompanied by the decline of Nitrate reductase expression, suggesting a change in nutrient allocation strategy. Nitrate absorbed by leaves under high herbivory and nutrients could be reallocated to other storage organs to make it inaccessible to herbivores, thus protecting N reserves needed to regrowth, and avoiding the enhancement of leaf nutritional quality.

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